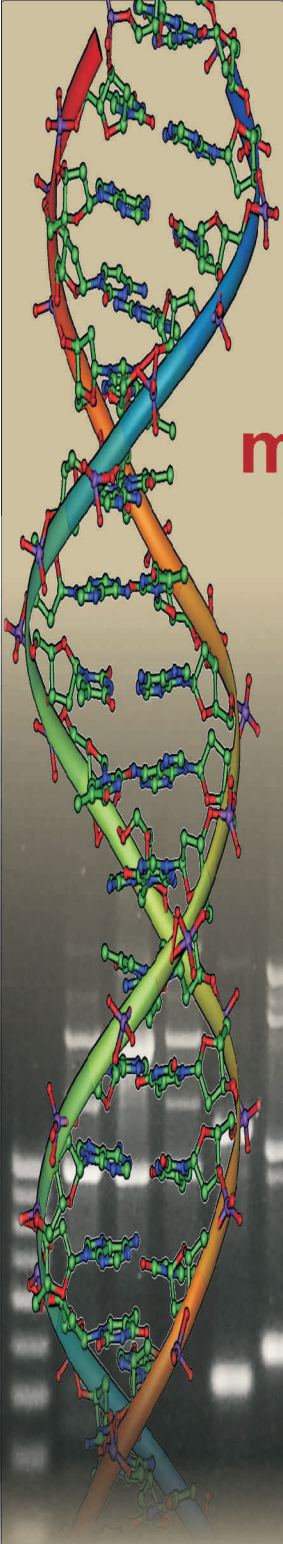
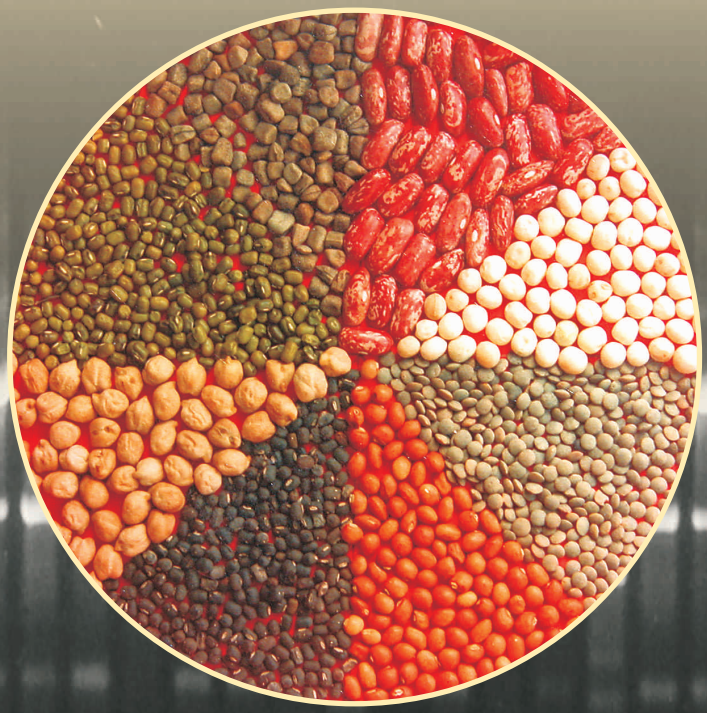
Technical Bulletin

DNA fingerprinting of major pulse crops of India





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DNA fingerprinting of major pulse crops of India

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Foreword

India is the largest producer as well as consumer of pulses in the world. It accounts for about 25 per cent of the global share. However, the growth in pulse production could not keep pace with the burgeoning population, resulting in the shortage of pulses in the country. In order to meet domestic requirements, we import 1.5-2.0 million tones pulses annually. To become self sufficient in pulses, we need to increase production at annual growth rate of 4.02%. This requires a sea change in the strategic research, technology generation and dissemination, and commercialization along with capacity building in frontier areas of research like genomics, transgenic development, bioinformatics and molecular breeding.

In India, a large number of pulse crop are being grown since ages. Indian national program in association with State Agricultural University and international institutions have developed more than 500 varieties of different pulse crops. The national gene bank conserves more than 34,218 accessions of different pulse crops. As a result of replacement of old varieties/land races by modern day varieties, there is loss of diversity globally which is a matter of great concern. In order to contain the loss of genetic diversity and sustain agricultural growth with maximum advantages, several steps are needed to be taken. The enactment of "Protection of Plant Varieties and Farmers Right Acts" (PPV & FR), 2001 and National Biodiversity Act 2002 are the important steps in this direction. For the purpose of safeguarding our interests and meeting obligation under TRIPs, India has opted for an effective "Sui generis" system for plant varieties protection. The country has already shown its preparedness as DUS (Distinctness, Uniformity and Stability) tests are already underway in twelve major crops of which six are pulse crops. The varietal characterization can be made more stringent by supplementing DUS results with DNA profile of concerned variety.

In order to characterize varieties/cultivars, data on DNA profiling was generated which will be helpful in identification of cultivar/variety, detection of duplicates, varietal registration and protection of plant breeders and farmers' right. In this publication, an effort has been made to obtain fingerprint data on important varieties of chickpea, pigeonpea, mungbean, lentil and fieldpea. I appreciate the sincere efforts made by Drs. P.K. Agrawal, P.R. Choudhary, S. Datta, N.P. Singh and Masood Ali for generating information on DNA fingerprint of important varieties of major pulse crops in present form. I am confident that this publication will be useful for plant breeders in selection of appropriate parents for hybridization, removal of duplicates in gene bank and registration of varieties for protection purposes.

October, 2007

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Preface

India produces a wide range of pulses, the major ones being chickpea, pigeonpea, urdbean, Mungbean, field pea and lentil. Although, the varietal improvement program in pulses are continuing through conventional breeding and hybrid technology in few cases, unlike major cereals, these crops have not benefited much through the advances in molecular breeding. This is mainly due to the lack of comprehensive information about the variability in the gene pool. The information on genetic characteristics of cultivars and germplasm lines and detection of duplicate accessions is an essential step to exploit genetic resources in breeding program.

With the introduction of Plant Variety Protection Act under GATT, the need of precise genotype characterization with clear Distinctness (D), Uniformity (U) and Stability (S) has attained greater importance. Therefore, proper documentation and genetic cataloguing of germplasm is becoming necessary in the era of patenting, intellectual property right and piracy of genetic materials. The developments of PCR (Polymerase Chain Reaction) based markers have opened new avenues for genotype identification. Along with the morphological and agronomical data, the DNA fingerprinting will be able to estimate genetic diversity and establish the identity of genotypes without any ambiguity. IIPR being the premier national institute on pulses research, the institute has the responsibility to characterize the germplasms of mandate crops.

We express our sincere gratitude's to Dr. Mangala Rai, Secretary, DARE & DG, ICAR, Dr. S.P. Tiwari / Dr. P. L. Gautam, DDG (Crop Sciences), ICAR, for their constant guidance and encouragement in bringing out this publications. An effort has been made to fingerprint the released varieties of the major pulse crops which will go a long way to help breeders and biotechnologists to identify the suitable materials for hybridization and also will offer a platform for establishing identity of our indigenous pulses germplasm.

October, 2007

Authors

Introduction

Pulses are the second most important group of crops after cereals. In 2005, the global pulse production was 61.72 million tones which come from 73.2 million ha with an average productivity of 843 kg/ha (2005-06). Dry bean contributed 31% to global pulse production followed by dry pea (18.3%), chickpea (14.9%), broad bean (7.5%), lentil (6.5%), cowpea (6%) and pigeonpea (5.3%). The annual growth rate in area and productivity was 0.87% and 1.83%, respectively. India, China, Brazil, Canada and Myanmar are the major pulse producing countries with relative share of 25%, 10%, 5%, 5% and 4%, respectively.

In India, pulses continue to be an integral part of Indian agriculture as they are an excellent and cheapest source of dietary protein and sustain crop production in fragile environment of rainfed areas. Despite release of large number of high yielding varieties, total production of pulses remained almost static between 13 to 15 million tones over the last 15 years mainly due to narrow genetic base, lack of genetic variability in useable gene pool, susceptibility to large number of biotic and abiotic stresses, photoperiod sensitivity, non synchronous flowering and maturity, low input use, huge post harvest losses and poor adoption of technology.

Conventional methods of crop improvement have not improved productivity of pulses significantly due to several reasons. These methods are slow and time consuming. Besides, lack of reliable resistant sources, undesirable linkages and long gestation period required for development of a variety are considered to be the main limitation of these methods. Biotechnology and genetic engineering has emerged as a potential tool in recent years for genetic manipulation of crop plants. The said technology promises to improve crop productivity through complimenting traditional breeding by decreasing dependence on harmful chemical pesticides, fertilizers and antibiotics etc. The tools of biotechnology are now available to plant breeders and can be used effectively to supplement conventional practices in crop improvement.

In pulse crops, a large number of land races, farmer's variety and scientifically developed cultivar existed in the 19th and early part of the 20th century and much of it have been replaced by new and better varieties. This large scale loss of variability that existed globally is a matter of concern even though loss of diversity does not mean by loss of genes. In order to contain the loss of genetic diversity and sustain agricultural growth and to position India to get maximum advantage, several steps need to be taken up. The "Protection of Plant Varieties and Farmers Rights Acts" was passed by the Parliament in 2001 which is a significant leap forward. In the long run it would facilitate the growth of the seed industry and make available high quality seeds and planting materials to farmers. Implementation of Act will accelerate agricultural development and protect plant breeder's rights. Above all it will protect the rights of the farmers.

India has rich biodiversity of pulse crops as this region is also centre of origin of many pulse crops. Over 500 varieties of various pulse crops have so far been released, out of which 179 are released after 1990 for cultivation in the various agro-climatic zones. Many of these varieties have high yield potential, disease resistance as well as shorter maturity duration. Besides, country also possesses more than 34,218 accessions of germplasm of different pulse crops. These resources are needed to be properly characterized, conserved and utilized in breeding programme. Since, DUS test is already underway in many crops, there is an urgent need to generate data on DNA fingerprints of these varieties/germplasm specifically to address IPR issues.

India ratified the Agreement on Trade Related Aspects of the Intellectual Property Rights (TRIPs) and for purposes of safeguarding our interests and meeting our obligations under the TRIPs an ordinance to amend the Patent Act, 1970 was promulgated in 1994. India has the observer status in the UPOV for the protection of new varieties of plants with its main office at Geneva, Switzerland. The TRIPs agreement under 27(3)(b) gives an option to member countries to adopt an effective "sui generis" system for plant varieties protection. By adopting a system in the context of UPOV (1978), the PPV & FR Act, 2001 has been made as a widely acceptable registration system. For protection of plant varieties, DUS (Dictinctness, Uniformity and Stability) are pre-requisites now a days. This DUS test can be made more stringent by supplementing data of DNA profiling of varieties under test seeking protection.

Molecular marker technology has revolutionized the entire scenario of biological sciences and agriculture. DNA-based molecular markers have acted as versatile tools and have found their utility in various fields like taxonomy, physiology, embryology, genetic engineering, etc. They are no longer being looked upon as simple DNA fingerprinting markers in variability studies in plant and animal species. These markers are constantly being modified to enhance their utility and to bring about automation in the process of genome analysis. This has facilitated the development of marker-based gene tags, map-based cloning of agronomically important genes, variability studies, phylogenetic analysis, synteny mapping, marker-assisted selection of desirable genotypes etc. Thus, giving new dimensions to concerted efforts of breeding and marker-aided selection that can reduce the time span of developing new and better varieties. These DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analyzed objectively.

Over the last few decades, plant genomics has been studied extensively bringing about a revolution in this area. Molecular markers, useful for plant genome analysis, have now become an important tool in this revolution. During the early period of research, classical strategies of using phenotypic markers were employed in genetic analysis to determine inter- and intra-species variability and employing indirect selection for economic traits. In the past decade, however, molecular markers have very rapidly complemented the classical strategies

in cereal crops. Molecular markers include biochemical constituents (e.g. secondary metabolites in plants) and macromolecules viz., proteins and deoxyribonucleic acids (DNA). Analysis of secondary metabolites is, however, restricted to those plants that produce a suitable range of metabolites that can be easily analyzed and which can distinguish between varieties. These metabolites which are being used as markers should be ideally neutral to environmental effects or management practices. Hence, amongst the molecular markers used, DNA markers are more suitable and ubiquitous to most of the living organisms.

The utility of any marker system largely depends on genetic polymorphism which is classically defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. Although, DNA sequencing is a straightforward approach for identifying variations at a locus, it is expensive and laborious. Several techniques have, therefore, been developed in the past few years for visualizing DNA sequence polymorphism.

DNA Fingerprinting

The term DNA-fingerprinting was introduced for the first time by Jeffery (1985) to describe bar-code-like DNA fragment patterns generated by multilocus probes after electrophoretic separation of genomic DNA fragments. The emerging patterns make up a unique features of the analyzed individual and are currently considered to be their ultimate tool for biological individualization. Recently, the term DNA fingerprinting / profiling is used to describe the combined use of several single locus detection systems and are being used as versatile tools for investigating various aspects of plant genomes. These include characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, plant breeding and diagnostics.

DNA fingerprinting can unambiguously distinguish one individual from another with much higher probability. The tools of DNA fingerprinting can even distinguish twins. Since its discovery, DNA fingerprinting is being widely used in Europe and America for various purposes like pedigree analysis, establishment of paternity and other family relationships, and immigration checking etc. DNA fingerprinting has also been used in identifying and determining linkage of disease loci. It has also been helpful in identifying and cloning of defective gene(s). In plants, it has been useful in the molecular identification of the plant species or genotypes. The molecular profiles can be utilized to demonstrate the uniqueness of the plants. India is one of the most biodiverse nation in the world having very large number of flora and fauna species. Among plants, agro-economically important plants *per se* forms a major biological resource and considering the increasing demand and importance, it is imperative that available Indian plant genetic resources especially agro economically important plants should not only be conserved but also should be protected in terms of IPR at international convention on biological diversity.

Methods for DNA fingerprinting:

The DNA fingerprinting methods can broadly be classified into two categories**:** i**)** Hybridization based DNA fingerprinting, and ii) PCR based DNA fingerprinting.

Hybridization based

RFLP (Restriction fragment length polymorphism) is the most used approach where DNA polymorphism is analyzed through the use of selected probes (based on the presence of restriction sites on the genome under study). To obtain a typical DNA fingerprint, probes are used to create complex banding pattern by recognizing multiple DNA loci simultaneously. Each of these loci is characterized by more or less regular arrays of tandemly repeated DNA motifs that occur in different number at different loci. RFLP has been used for studying inter- and intra- specific genetic variations among the cultivars and wild population using random or specific DNA Probes. RFLP has been used to prepare chromosome maps in human, mice, fruit fly, tomato, sugar beet and rice.

PCR based

Polymerase Chain Reaction (PCR): It involves the *in vitro* amplification of particular DNA sequence with the help of specifically or arbitrarily chosen oligonucleotides primers and the thermo stable DNA polymerase, followed by electrophoretic separation of the amplified fragment. PCR for the first time was described in 1985 (Karry Mullis *et al.* 1985). It is a rapid technique that involves *in vitro* enzymatic amplification of target DNA segment, where the product of the first cycle became a template for next cycle. PCR has generated impact on various branches of modern biological sciences, including the field of genomics.

RAPD (Random Amplified Polymorphic DNA)

It was in 1990, two groups *viz*. Welsh and McClelland, and Williams *et al*. published modified PCR –based genetic assay that overcomes the drawback of the selective gene amplification. Their modification involved use of a single shorter primer (5 to 10 nucleotides) with GC content of 50%-80%. The nucleotide sequence was selected at random. This modified PCR-based genetic assay is known as Random Amplified Polymorphic DNA or RAPD (pronounced as rapid). The development of RAPD markers allows for the examination of genomic variation without prior knowledge of DNA sequence. This PCR based method does not involve labelling probes and requires small amount of relatively crude genomic DNA. In this analysis usually 10-mers or 20-mers primers generate amplification products (by PCR) and are subsequently separated on agarose gel electrophoresis in the presence of ethidium bromide and visualized against ultraviolet light (Williams *et al.* 1990). The genetic marker screening is based on the survey of genetic diversity as revealed by variation at

specific gene loci and provides information about the amount and distribution of genetic diversity within and among populations.

RAPD offers several advantages over other commonly used technique like RFLP. The RAPD technique is rapid and of low cost. RAPD technology facilitates the identification of a unique genotype from several others in a population. The RAPD technique has been used efficiently for the analysis of diversity, phylogenetic relationship, identification of gene, establishment of core collection and identification of duplicates within the largest germplasm collection. This technology is also found to be invaluable in plant pathogen analysis, especially those pathogens which cannot be grown easily *in vitro.* It provides potentially infinite number of variable markers for the development of genetic maps and has been used in crop improvement programmes in agricultural species.

RAPD markers are useful tool for fingerprinting of a genome. It has been used to determine the genetic variability in several medicinal and aromatic plants like Vetiveria, Mentha, Taxus and Bacopa. RAPD has also been applied to several crop plants to address genetic diversity issues such as in peas, beans, barley, wheat and alfalfa. These markers provide a level of resolution equivalent to RFLP and their congruence with other maker system has also been demonstrated.

SSRs (Simple Sequence Repeats)

The analysis of microsatellite DNA loci is the current method of choice for population analyses. Microsatellite loci consist of short (2-6 bp) tandemly-repeated nucleotide arrays surrounded by unique flanking sequences. These loci are distributed throughout the genome in high abundance. It is estimated that the mammalian genome may contain in excess of 100,000 to 300,000 such loci or one locus every 10-30 kilobase pairs. Allelic diversities and heterozygosities are typically extremely high in SSRs and the presence of 10 or more alleles, and heterozygosities in excess of 0.85 are not uncommon. Retrieval of SSRs has not been easy in plants because of their relative low abundance compared with animal genomes (Gupta *et al.* 1994). Where they have been isolated, it has often been found that they show limited cross transferability to other genera and even to other species within the same genus. All this means that the investigator wishing to choose SSRs is first faced with having to isolate them. Microsatellite sequences are especially suited for distinguishing between closely related genotypes and are therefore favoured in population studies and for identification of closely related cultivars. SSRs have been shown to provide a powerful means for discrimination between closely related genotypes in many plant species.

Following domestication, genetic variation in crop plants has continued to narrow down due to continuous selection pressure for specific traits i.e. yield, thus rendering them more vulnerable to disease and insect epidemics and jeopardizing the potential for sustained genetic

improvement over a long term .Thus it is extremely important to study the genetic composition of the germplasm of existing modern-day cultivars in comparison with their ancestors and related species. This will not only provide information on their phylogenetic relationship but will also indicate a chance of finding new and useful genes, as the accessions with most distinct DNA profiles are likely to contain a greater number of novel alleles. DNA profiling to make such sampling decisions is now underway in most crops in India.

Among the various molecular markers, PCR based RAPD markers have become popular because their application does not need any prior information about the target sequences on the genome. RAPD has been the most employed technique because it is simple and fast. Despite questions about its reproducibility, its utility in diversity analysis, mapping and genotype identification has been exploited in many plant species.

Many DNA markers both specific as well as arbitrary have been used so far, for DNA fingerprinting of various classes of germplasm. Further studies with STMS (Sequence Tagged Micro Satellite) markers may also throw light on the domestication process involved in crop plants and provide useful criteria for enriching the gene pool of crop plants and determine how efficient plant breeders have been in accessing pre-existing forms of variation. AFLP (Amphid Fragment Length Polymorphism), a new class of molecular markers, has gained popularity as marker for the study of genetic polymorphism especially in species where polymorphism is extremely rare using other types of markers. Similarly the potential of ISSR markers has been exploited for diversity analysis of number of crop plants. These studies have helped in the classification of existing biodiversity among plants, which can be further exploited in gene introgression from wild genera and species.

The repetitive and arbitrary DNA markers are markers of choice in genotyping of cultivars. Microsatellites like (CT) 10, (GAA) 5, (AAGG) 4, (AAT) 6 (123), (GATA) 4, (CAC) 5 and minisatellites have been employed in DNA fingerprinting for the detection of genetic variation, cultivar identification and genotyping. This information is useful for quantification of genetic diversity, characterization of accessions in plant germplasm collections and taxonomic studies. Microsatellites have been useful for generation of STMS markers, revealing polymorphisms within closely related cultivars. The first application of microsatellites in plants has been in cultivar identification, wherein microsatellites have been used to genotype unequivocally diverse materials like rice, wheat, grapevine (*Vitis vinifera*), soybean etc. This is important especially for protection of proprietary germplasm. Similarly microsatellite markers have also been advantageous in pedigree analysis as they represent single locus. The multi allelism of these markers facilitates comparative allelic variability detection reliably across a wide range of germplasm and allows individuals to be ubiquitously genotyped, so that gene flow and paternity can be established. One of the most recent applications of these markers has been shown in sex identification of dioeceous plants, wherein microsatellite probe (GATA) 4 is found to reveal sex-specific differences in Southern analysis and can be used as a diagnostic marker in this system where male and female plants do not show any sex-specific morphological difference until flowering.

PIGEONPEA

Background

Pigeonpea is one of the major pulse crop of the tropics and subtropics and occupies the second position in terms of area and production in India after chickpea. India shares 90 percent of world's area and production in pigeonpea. It accounts for 3.56 million hectares of area and 2.62 million tones of production (FAO, 2006). Among the variety of usage of pigeonpea, the dry seeds are cooked to make thick soup (*dhal*). The protein content of pigeonpea seed is comparable with any pulse crop and ranges between 18.5 to 26.3%. The seed husks, pod walls and green leaves are used as cattle feed and leaves are also used to feed silk worms. Being a legume, it fixes nitrogen. The leaf fall at maturity not only adds to the organic matter in the soil, but also provides additional nitrogen.

Estimation of genetic diversity and its exploitation in breeding program is essential for crop improvement. Widely adopted PCR based marker technologies such as RAPD, ISSR and STMS amplify different regions of the genome as they are dispersed across the plant genome and have their own advantages and disadvantages. RAPD possess the simplest and fastest detection technology, identifies multi locus markers and used in numerous crops species for detecting genetic diversity including pulses. So far only a few attempts have been made to characterize the genetic diversity in pigeonpea by using DNA markers. We assessed the genetic diversity and relationships of popular cultivars/advance breeding lines developed in different national and international research institutes. These genotypes possess the capacity to grow in the tropical and sub-tropical region across the globe. These genotypes are widely adapted, popular and high yielding cultivar of pigeonpea. Most of the selected genotypes are originated from diversified pedigree, possess various geographical distribution; having different morphological character, maturity time and growth habit. RAPD has been used for diversity analysis in several crop plants including legumes. Therefore, we assayed the efficiency of the RAPD marker system to asses the genetic variability among the pigeonpea gene pool. In this context, unique bands produced by pigeonpea genotypes with specific RAPD primers were scored and documented for their precise identification.

Plant material

A total of 24 elite cultivars and advanced breeding lines of pigeonpea representing large morphological variations, maturity group, agro climatic zones and parentage were selected for genetic diversity studies. All the cultivars and advanced breeding lines used in the present study have been developed from National and International research institutes/universities located in India (Table 1). All of these materials were collected from core collection maintained by the pigeonpea breeders at Indian Institute of Pulses Research, Kanpur, India.

Table 1 : Brief information about pigeonpea cultivars used

S. No.		Pedigree	Morphological characters	Resistance to biotic stress**	Institution where developed*
1.	UPAS 120	Selection from P 4768	Semi spreading & indeterminate	Resistant to *Fusarium* wilt	GBPUA&T, Uttaranchal, India
2	MAL 6	MA-2 X Bahar	Spreading	Resistant to SMD	BHU ($U.P^{+}$., India)
3.	MAL 13	(MA-2 X MA 166) X Bahar	Spreading	Resistant to SMD	BHU (U.P., India)
4.	PDA 10	Local Selection from Akbarpur, Kanpur Dehat (U.P.)	Compact & erect, indeterminate, large seeds	Resistant to SMD	IIPR (U.P., India)
5.	PDA 92-1	Bahar x ICP 8863	Spreading, indeterminate yellow flowers, small seeded, high yielding	Resistant to SMD and *Fusarium* wilt	IIPR (U.P., India)
6.	IPA 402	Local selection from Jaunpur (U.P.)	Semi Spreading	Resistant to SMD	IIPR (U.P., India)
7.	IPA 602	Bahar x ICPL 84023	Compact & erect, large seeded	Resistant to SMD	IIPR (U.P., India)
8.	IPA 3-1	Bahar X ICPL 96058 (ICRISAT line)	Compact & erect	Resistant to SMD	IIPR (U.P., India)
9.	IPA 3-2	Bahar X ICPL 96058 (ICRISAT line)	Compact & erect	Resistant to SMD & *Fusarium* wilt	IIPR (U.P., India)
10.	ICPL 84023	Selection from ICRISAT germplasm lines	Semi spreading and determinate	Resistant to SMD, *Fusarium* wilt and PSB	ICRISAT (A.P., India)
11.	ICPL 88039	Selection from ICRISAT germplasm lines	Semi spreading, early in maturity, tolerant to water logging	Resistant to SMD	ICRISAT (A.P., India)

12.	ICP 8863	Selection from land race of Maharashtra	Spreading, indeterminate, yellow flowers, medium seeded	Resistant to *Fusarium* wilt	ICRISAT (A.P., India)
13.	ICPL 87119	C-11 X ICPL 6	Indeterminate, spreading	Resistant to *Fusarium* wilt and SMD	ICRISAT (A.P., India)
14.	Pusa 9	UPAS 120 X 3673	Indeterminate & erect	Resistant to SMD and alternaria blight	IARI (New Delhi, India)
15.	Pusa 992	Selection from ICPL 90306	Indeterminate, early maturity, semi spreading	Resistant to *Fusarium* wilt	IARI (New Delhi, India)
16.	CO 5	Mutant of Co 1	Semi spreading and bushy	Moderately resistant to SMD	TNAU (T.N., India)
17.	CO 6	Mutant of SA 1	Indeterminate, semi spreading	Tolerant to pod borer	TNAU (T.N., India)
18.	BMSR 853	(ICP 7336 X BDN-1) X BDN-2	Spreading, large and white seeded	Resistant to *Fusarium* wilt and SMD	ARS, Badnapur, (M.S., India)
19.	Amar	Selection from Bahar	Indeterminate and erect growth habit	Resistant to SMD	CSAUA&T, Kanpur, (U.P., India)
20.	Bahar	Selection from land race of Motihari, Bihar	Indeterminate, compact & erect, yellow flower, large seeded	Resistant to SMD	RAU, Pusa, (Bihar, India)
21.	T-7	Selection from land race of Lucknow	Compact, erect, tall high yielding with large seeded	Susceptible to wilt and SMD	CSAUA&T, Kanpur, (U.P., India)
22.	DA-11	Bahar x NP (WR) 15	Compact & erect	Resistant to SMD and alternaria blight	RAU, Pusa, (Bihar, India)
23.	NDA-1	Selection from land race of Faizabad, (U.P.)	Indeterminate, compact & erect, high yielding	Resistant to SMD, tolerance to wilt	NDUA&T, Faizabad, (U.P. India)
24.	KPL 43	Selection from Bahar	Indeterminate, compact & erect	Resistant to *Fusarium* wilt, SMD and PSB	IIPR, Kanpur, (U.P., India)

*Abbreviation of the institutions/universities:

BHU: Banaras Hindu University; IIPR: Indian Institute of Pulses Research; ICRISAT: International Crops Research Institute for the Semi-Arid Tropics; IARI: Indian Agricultural Research Institute; TNAU: Tamil Nadu Agricultural University; ARS: Agricultural Research Station; CSAUA&T: Chandra Shekhar Azad University of Agriculture and Technology; RAU: Rajendra Agricultural University; NDUA&T: Narendra Deva University of Agriculture and Technology; GBPUA&T: Govind Ballabh Pant University of Agriculture and Technology

*Abbreviation of the states of India:

U.P.= Uttar Pradesh; M.P.= Madhya Pradesh; A.P.= Andhra Pradesh; M.S.= Maharashtra; T.N.= Tamil Nadu

Abbreviation of the biotic stress**:

SMD = Sterility Mosaic Disease; PSB = Phytophthora Stem Blight

Methodology

DNA extraction

Genomic DNA from each pigeonpea germplasm was extracted from bulked leaf samples (approximately 1 g each) plucked from one month old young seedlings. Isolation of DNA was done based on the modified protocol of Guillemant & Laurence (1992). Pooled leaf samples were ground to a very fine paste using the grinding buffer [100 mM sodium acetate, pH 4.8; 500 mM NaCl; 50 mM EDTA, pH 8.0; 50 mM Tris, pH 8.0; 2% PVP (MW 10000); 1.4% SDS] and incubated at 65°C for 30 minutes. Then 0.6 volume of the 10M Ammonium acetate was added into each tube and kept for another 15 minutes at 65^{0}C followed by centrifugation at 10K rpm for 10 minutes. Supernatant was taken out and 0.6 volume of chilled Iso-propyl alcohol was added and kept at –20^{0}C for 60 minutes for better precipitation of DNA. DNA was pelleted out by centrifugation, washed twice with 70% ethanol and dissolved in TE buffer (10 mM Tris, 1.0 mM EDTA, pH 8.0). Dissolved DNA solution was extracted with Phenol: Chloroform: Iso-amyl alcohol (25:24:1) and RNA was removed by RNase treatment (@ 4 µl/ml of supernatant from stock of 10mg/ml of RNAse) at 37 ^{0}C for 1 hr. RNAse treated DNA was further extracted twice with Chloroform: Iso-amyl alcohol (24:1) for further purification. DNA was re-precipitated in chilled ethanol (100 %) and dissolved in TE buffer. Purified DNA was checked for its quality and quantity by 0.8% agarose gel

electrophoresis using uncut lambda (λ) DNA as standard marker (300 ng/µl). Dilution of the DNA solution was done using TE buffer to a concentration of approximately 12.5 ng/ µl for use in PCR analysis.

DNA amplification by PCR

PCR amplification was carried out in 0.2 ml thin-wall PCR tubes using a MJ Research DNA Engine thermal cycler (model PTC 200). A total of 100 RAPD primers (20 primers each from series OPX, OPAQ, OPAZ, OPH and OPP of Operon Technologies, Alameda, CA, USA) were screened in our present study and unambiguous DNA profile was generated by 76 primers (Table 2). Polymerase chain reaction (PCR) mixture of 25 µl contained 25 ng of genomic DNA template, 0.6 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 0.3 µM of decamer primer, 2.5 µl of 10 X PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$) and 0.25 µl of pooled dNTPs (100 mM each of dATP, dCTP, dGTP and dTTP from Fermentas Life Sciences, USA). PCR cycle conditions were as follows: initial denaturing step at 94°C for 3 min followed by 44 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min. In the last cycle, primer extension at 72°C for 7 min was provided.

Documentation of Agarose gels

PCR products were separated through electrophoresis on a 1.5% agarose gel containing ethidium bromide using 1X TBE buffer (pH 8.0). The amplified products were visualized and photographed under UV light source. O'Gene Ruler ™ 100 bp DNA Ladder Plus (Fermentas Life Sciences, USA) was used as molecular weight marker.

Analysis of data

DNA bands were scored '1' for its presence and '0' for its absence for each primer genotype combination. These binary data matrix was then utilized to generate genetic similarity data among genotypes. Only unambiguous bands were scored for the estimation of genetic similarity between the varieties using Jaccard's similarity coefficient. Based on these data, UPGMA (unweighted pair group method using arithmetic averages) clustering was carried out by applying the software NTSYS-pc (Rohlf 1992). Support for clusters was evaluated by bootstrap analysis with Win Boot software (Yap and Nelson 1995). One thousand samples were generated by re-sampling with replacement of characters within the combined 1/0 data matrix. Multidimensional 2D plot analysis was done to compare and support the clustering pattern obtained by UPGMA dendrogram. The expected heterozygosity for a genetic marker (Hn) was calculated by $Hn = 1\text{-}pi^2$ (pi is the allele frequency of the ith allele; Nei, 1987). By using the values of Hn, Hav (the arithmetic mean heterozygosity) was calculated by Hav = Hn/n (n = number of markers or loci analysed, Powell *et al.* 1996). The average heterozygosity for polymorphic markers (Hav) p was derived as (Hav)p = Hn/np (np = no. of polymorphic

Table 2. Analysis of the amplified DNA fragments obtained with 76 RAPD primers in various cultivars of pigeonpea

S.No.	Primer name	No. of amplified band	Polymor-phic band	Monomor-phic band	No. and mol. wt. of unique band in base pairs (bp) with serial no. of variety
1.	OPX 01	7	4	3	0
2.	OPX 02	6	4	2	0
3.	OPX 03	10	8	2	1(2000bp-1)
4.	OPX 04	10	8	2	2 (800bp-4, 700bp-18)
5.	OPX 06	8	7	1	0
6.	OPX 11	12	8	4	0
7.	OPX 12	9	5	4	1(1500bp-23)
8.	OPX 13	6	1	5	0
9.	OPX 14	5	4	1	0
10.	OPX 15	2	1	1	0
11.	OPX 17	2	1	1	0
12.	OPX 19	5	5	0	0
13.	OPAQ 04	15	12	3	1(1350 bp-16)
14.	OPAQ 05	19	9	10	3 (1100bp-18,1031bp-18,425bp-18)
15.	OPAQ 06	11	0	11	0
16.	OPAQ 07	12	11	1	0
17.	OPAQ 08	6	2	4	0
18.	OPAQ 09	9	8	1	1(500bp-10)
19.	OPAQ 12	15	4	11	0
20.	OPAQ 13	14	4	10	0
21.	OPAQ 14	12	10	2	0
22.	OPAQ 15	17	13	4	0
23.	OPAQ 16	9	5	4	1(430bp-24)
24.	OPAQ 18	21	20	1	3(3100,2500,425bp-12)
25.	OPAQ 19	20	19	1	5(2400,2200,1400,1300,500bp-12)
26	OPAZ 01	9	8	1	0
27	OPAZ 03	7	6	1	1(700bp-21)
28	OPAZ 04	8	6	2	0
29	OPAZ 05	10	9	1	2(2200bp-1,1900bp-1)
30	OPAZ 08	11	8	3	0
31	OPAZ 09	12	5	7	0
32	OPAZ 11	10	6	4	1(750bp-21)
33	OPAQ 20	18	14	4	1(400bp-14)
34	OPAZ 12	11	5	6	0
35	OPAZ 14	5	4	1	0
36	OPAZ 16	9	4	5	1(900 bp-24)

(Contd .....)

S.No.	Primer name	No. of amplified band	Polymor-phic band	Monomor-phic band	No. and mol. wt. of unique band in base pairs (bp) with serial no. of variety
37	OPAZ 18	14	14	0	2(1500 bp-23,900bp-15)
38	OPAZ 19	7	7	0	0
39	OPAZ 20	7	5	2	0
40	OPH 01	13	13	0	2(1950 bp-18,900 bp-1)
41	OPH02	10	9	1	0
42	OPH 03	14	12	2	2(800bp-22,625bp-22)
43	OPH 04	10	8	2	0
44	OPH 05	8	5	3	0
45	OPH07	8	7	1	0
46	OPH 08	10	4	6	0
47	OPH 09	16	15	1	0
48	OPH 10	11	10	1	2(2000bp-21,1150bp-9)
49	OPH11	17	16	1	1(2500bp-5)
50	OPH 12	11	5	6	2(1350bp-18,1300bp-18)
51	OPH 13	11	7	4	0
52	OPH 14	9	8	1	0
53	OPH 15	8	5	3	0
54	OPH 16	2	1	1	0
55	OPH 17	12	9	3	2(1250bp-8,700bp 12)
56	OPH 18	8	5	3	0
57	OPH 19	8	4	4	0
58	OPH 20	2	2	0	0
59	OPP 01	4	2	2	0
60	OPP 02	9	8	1	1(2600 bp-15)
61	OPP 03	9	9	0	1(910 bp-7)
62	OPP 04	15	15	0	3(600 bp -21,520 bp -8,500 bp -11)
63	OPP 05	15	14	1	3(1200 bp -17,800 bp -21,780 bp -15)
64	OPP 06	10	4	6	1(550 bp -18)
65	OPP 07	11	9	2	2(1400 bp -23,1200 bp -23)
66	OPP 08	17	15	2	1(350 bp -20)
67	OPP 09	14	12	2	1(1450 bp -5)
68	OPP 10	19	17	2	3(2150 bp -5, 2000 bp -18,1300 bp -5
69	OPP 11	10	9	1	0
70	OPP 12	16	10	6	0
71	OPP 13	10	5	5	0
72	OPP 14	15	10	5	1(2000bp-12)
73	OPP 15	12	10	2	0
74	OPP 16	8	7	1	0
75	OPP 17	7	6	1	0
76	OPP 19	7	5	2	1(1100 bp-19)

markers or loci). Marker Index (MI) was also calculated as MI = E (Hav)p (E is effective multiplex ratio and measured by nâ where â is the fraction of polymorphic marker or loci).

Significant Findings

RAPD Polymorphism

- Out of total 100 primers tried in the PCR amplification, 76 primers showed clear and unambiguous amplification while rest of the primers did not give amplification or produced faint or fuzzy lanes. Scorable 76 RAPD primers led to amplification of 796 fragments ranging from about 4700 bp (by OPP 14) to 250 bp (OPAQ 18), out of which 586 (73.4%) bands were found to be polymorphic.
- The level of polymorphism ranged from 0 % (by OPAQ 06, where all the 11 bands found to be monomorphic) to 100% (by OPX 19, OPAZ 18,19, OPH 01, 20 and OPP 03, 04 where the number of amplified DNA fragments were found to be 05, 14, 07, 13, 02, 09 and 15, respectively). Maximum number of 21 amplified products were obtained by primer OPAQ 18. Moreover, OPAQ 19 produced 20 bands followed by OPP 10, OPAQ 05 with 19 bands each. A minimum of 2 bands each was amplified by the primers OPH 16 and OPH 20 both.
- On an average 10.47 bands per primer were obtained and 33 primers out of 76 primers (43.4%) used in the study produced amplified products more than the average value of 10.47.
- Fifty five RAPD products produced by 32 RAPD primers were recorded as genotype specific. DNA amplification pattern as detected by some of the RAPD primers in the elite pigeonpea genotypes has been provided in Fig. 1 (a,b,c,d).

Heterozygosity and marker index

- Heterozygosity was calculated for the 796 amplified products obtained by employing 76 RAPD primers across the varieties. The Hav and (Hav) p were found to be 0.48 and 0.652, respectively, whereas the marker index (MI) value was obtained to be 5.027.

Genetic relationship and genotyping

- The degree of genetic relatedness among genotypes evolved from various geographical areas, morphological parameters, maturity groups and parentages varied considerably (0.567 to 0.827)(Table 3). Highest similarity (0.827) was measured between IPA 602 and ICPL 84023. Very high degree of similarity was also found between CO5 and CO6; PDA 10 and IPA 602; IPA 3-1 and IPA 3-2 and ICPL 84023 and Pusa 992. Least similarity (0.567) was found between semi spreading, determinate ICPL 84023 and

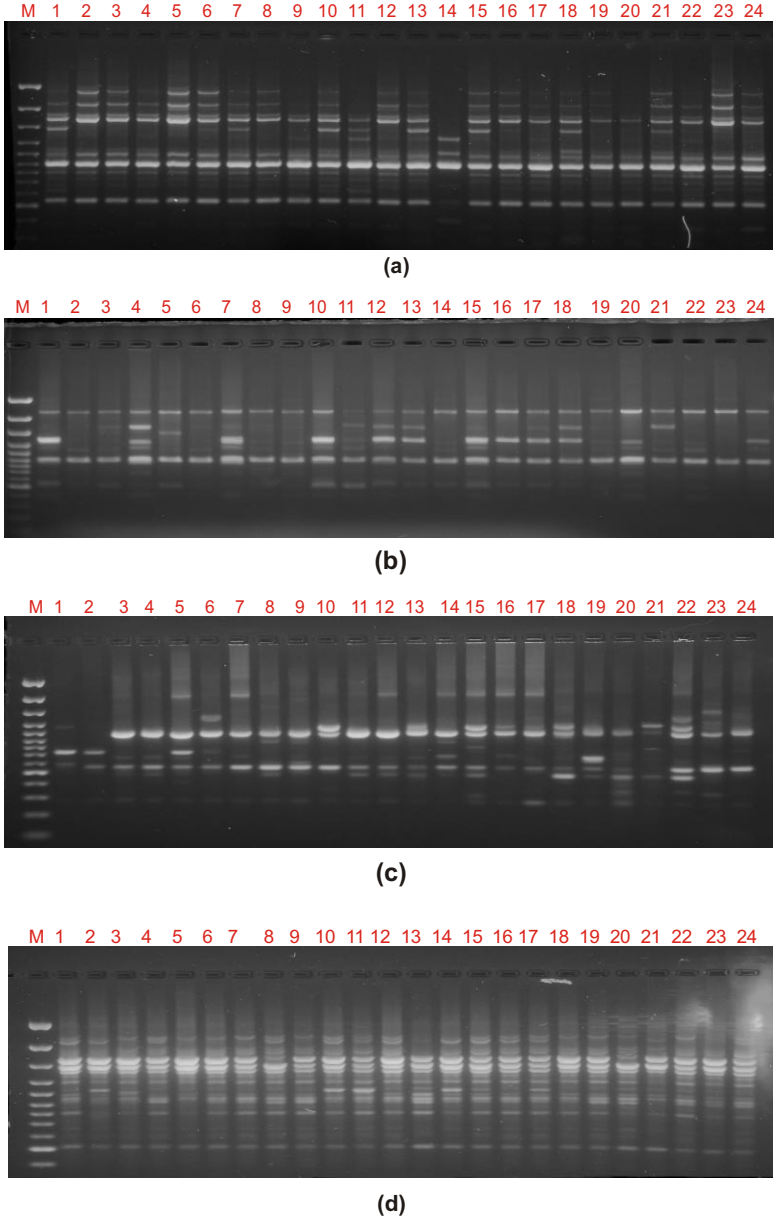


Fig. 1(a, b, c, d). DNA profile of pigeonpea cultivars obtained with RAPD primers OPAQ 20 (a), OPP 09 (b), OPAZ 18 (c) and OPH 13 (d). Serial number of the varieties corresponds to table 1. M=Standard DNA marker, 100 bp DNA ladder plus

Table 3. Genetic similarity matrix of the pigeonpea cultivars. The digits show the genetic relatedness among a pair of genotype. Serial no. of the genotypes (1-24) is as given in the table 1.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	1.000																						
2	0.719	1.000																					
3	0.656	0.771	1.000																				
4	0.755	0.725	0.730	1.000																			
5	0.657	0.766	0.767	0.713	1.000																		
6	0.696	0.781	0.759	0.751	0.767	1.000																	
7	0.750	0.709	0.700	0.812	0.730	0.759	1.000																
8	0.683	0.756	0.744	0.784	0.739	0.783	0.785	1.000															
9	0.699	0.697	0.678	0.742	0.700	0.726	0.759	0.812	1.000														
10	0.761	0.670	0.664	0.785	0.671	0.712	0.827	0.753	0.748	1.000													
11	0.688	0.694	0.676	0.711	0.688	0.698	0.734	0.765	0.769	0.760	1.000												
12	0.705	0.655	0.649	0.749	0.658	0.685	0.753	0.701	0.707	0.735	0.702	1.000											
13	0.674	0.680	0.657	0.680	0.672	0.673	0.712	0.704	0.729	0.696	0.720	0.708	1.000										
14	0.668	0.709	0.677	0.717	0.677	0.684	0.714	0.746	0.726	0.715	0.734	0.694	0.753	1.000									
15	0.753	0.683	0.649	0.773	0.683	0.731	0.789	0.726	0.693	0.807	0.716	0.758	0.728	0.754	1.000								
16	0.678	0.716	0.698	0.701	0.710	0.712	0.737	0.764	0.738	0.708	0.729	0.689	0.762	0.746	0.751	1.000							
17	0.694	0.746	0.723	0.723	0.731	0.744	0.755	0.774	0.763	0.712	0.749	0.716	0.759	0.755	0.736	0.823	1.000						
18	0.659	0.690	0.681	0.676	0.676	0.704	0.696	0.720	0.669	0.675	0.684	0.674	0.713	0.684	0.712	0.787	0.757	1.000					
19	0.679	0.727	0.708	0.725	0.729	0.734	0.753	0.778	0.752	0.709	0.748	0.696	0.734	0.754	0.732	0.754	0.784	0.747	1.000				
20	0.707	0.687	0.670	0.751	0.676	0.712	0.765	0.750	0.703	0.728	0.701	0.715	0.684	0.698	0.755	0.699	0.715	0.691	0.773	1.000			
21	0.575	0.614	0.609	0.597	0.615	0.637	0.607	0.608	0.574	0.567	0.570	0.568	0.577	0.581	0.599	0.582	0.598	0.602	0.631	0.616	1.000		
22	0.665	0.679	0.685	0.699	0.688	0.689	0.716	0.744	0.721	0.686	0.730	0.679	0.714	0.736	0.698	0.711	0.748	0.700	0.774	0.726	0.606	1.0 00	
23	0.619	0.702	0.656	0.648	0.693	0.713	0.641	0.696	0.658	0.618	0.633	0.619	0.603	0.637	0.632	0.650	0.680	0.642	0.691	0.645	0.644	0.648	1.000
24	0.674	0.730	0.708	0.726	0.733	0.732	0.739	0.761	0.722	0.706	0.707	0.696	0.720	0.739	0.707	0.758	0.769	0.727	0.775	0.727	0.609	0.762	0.692	1.000

compact, erect, tall T7. It was also observed that T7 showed considerable amount of diversity with ICPL 8863 and UPAS 120. NDA-1 was also found to show high diversity with ICPL 87119 and with few more genotypes.

- Multivariate (cluster) analysis of the genetic similarity data grouped the cultivars into two major clusters (I and II). Cluster I consists of two sub-clusters (Ia and Ib). Sub-cluster Ia consists of seven genotypes where four genotypes (PDA 10, IPA 602, ICPL 84023 and Pusa 992) were present in the core cluster. Sub-cluster Ib again comprised of 4 sub-groups (i, ii, iii and iv), with a total of 11 genotypes and cluster II consists of four genotypes. However, two cultivars (NDA-1 and T7) showed considerable diversity and could not be included in any cluster (Fig. 2).
- Bootstrap analysis was used to evaluate the degree of support for clusters within the dendrogram. It was observed that clusters, sub-clusters and sub-groups within the dendrogram were supported by high bootstrap values. It was further observed that both the multi-dimensional 2D plot (Fig. 3) and UPGMA dendrogram depicted similar clustering pattern.

Identification of pigeonpea genotypes

- RAPD analysis using a substantial scorable amount of 76 primers could generate a very large number of amplified products good enough to identify distinct banding pattern pertaining to specific cultivar/breeding line. Out of these RAPD primers, 32 primers produced 55 products that were recorded as genotype specific (Table 2).

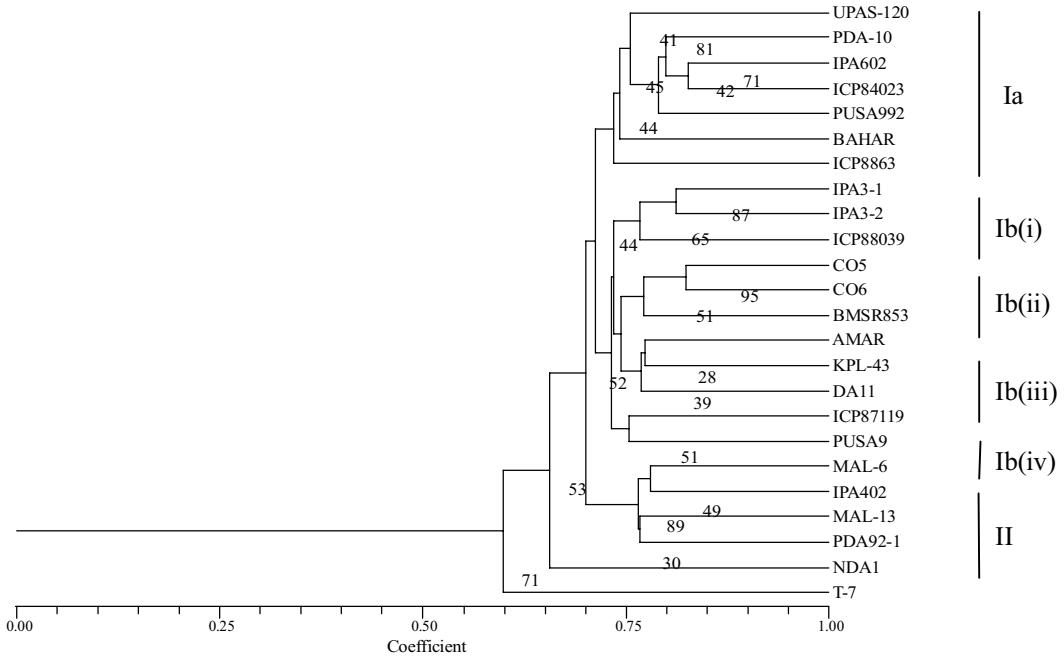


Fig. 2. Dendrogram of pigeonpea varieties constructed using UPGMA based on 76 RAPD primers. The major clusters and sub-clusters are indicated on right margin. Numbers at branch points indicate support for varieties clustered; values are percent of bootstrap sample that exhibited the cluster (no number at branch indicates support less than 10%). The major clusters, sub-clusters and sub-groups are indicated on right margin

Detection of polymorphism

- In the present study, 73.4% fragments were found to be polymorphic and an average of 10.47 bands per primer. Ratnaparkhe *et al.* (1995) obtained 7.93 bands per primer using 16 polymorphic RAPD primers in 10 cultivars. Moreover, estimated genetic similarity obtained by the same workers varied from 0.7 to 0.9, whereas in present studies it varied from 0.567 to 0.827 because of the wide range of geographical distribution, maturity group and different pedigree of the selected genotypes.
- RAPD has been found to be well correlated with other marker systems in identifying genetic diversity in pigeonpea. The range of genetic diversity using 76 RAPD primers was found to be higher (0.567 to 0.827) as compared to a combination of AFLP primers (a total of two Eco RI and 15 MseI), where the value was observed to be 0.82-1.00 with little polymorphism of 13.28% in 20 cultivars, thus indicating RAPD as an efficient marker system.

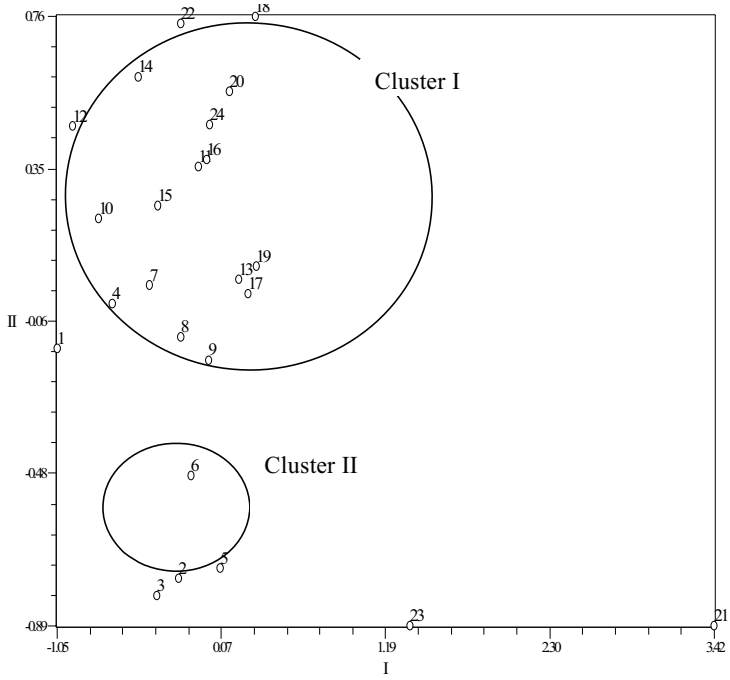


Fig. 3. Multidimensional 2D plot of pigeonpea genotypes generated from RAPD data. Both the cluster I and II depicts closely related genotypes as comparable to dendrogram. Serial number of the genotypes is as given in table 1. The scale shows various coordinate levels

- The Hav value was found to be higher (0.652), whereas the marker index (MI) was obtained to be 5.027and thus proving the efficiency of RAPD as a marker system in detecting heterozygosity in pigeonpea. The estimated mean heterozygosity (Hav) was found to be less in self pollinating leguminous crop cowpea (0.027; Pasquet, 2002) and wild lentil (0.342; Huh & Huh, 2001), based on allozyme diversity. When RAPD was used as marker system, the Hav value was found to be 0.203 in *Trigonella foenum–graecum* and 0.346 in *Trigonella caerula* (Dangi *et al*. 2004).

Phylogenic relationship

Earlier protein and isozymes analysis was carried out to estimate variability in pigeonpea cultivars (Ladizinsky and Hamel 1980). The major drawback of these techniques is presence of limited polymorphism among the closely related cultivars. Earlier RAPD (Ratnaparkhe *et al*. 1995) and AFLP (Panguluri *et al*. 2006) was used to detect genetic variability among pigeonpea cultivars.

- The present study, using a total of 76 RAPD primers, developed altogether 796 bands and among them 586 were found to be polymorphic (73.4%). The average similarity index of 75.0 between cultivars suggests that the genetic diversity detected by RAPD among the pigeonpea cultivars is higher as compared to former works done in pigeonpea diversity analysis.
- The average number of bands developed (10.47) and the average percent polymorphism (73.4) indicated the efficiency of RAPD primers in detecting polymorphism among cultivars. Highest similarity (0.827) was measured between IPA 602 and ICPL 84023 because ICPL 84023 is one of the parents of IPA 602 (Bahar x ICPL 84023). Least similarities were obtained between tall, compact, erect land race from northern India (Lucknow) T 7 with semi-spreading, determinate ICRISAT line ICPL 84023 and again between T 7 with spreading, intermediate ICP 8863 (a land race from western part of India). Furthermore, one more compact, erect and intermediate land race from northern part of India (Faizabad) NDA-1 showed significant variations with spreading, intermediate type ICPL 87119 and semi-spreading, determinate type ICPL 84023.
- The dendrogram is constituted of two major clusters and the robustness of the dendrogram as assessed by the bootstrap procedure, further supported the ability of RAPD to represent the genetic structure of the collection. It was further observed that both the multi-dimensional 2D plot and UPGMA dendrogram gave a similar clustering pattern of the genotypes studied using 76 RAPD primers, thus supporting the usefulness of RAPD in detecting molecular diversity.
- Cluster Ia consists of seven genotypes viz. UPAS 120, PDA 10, IPA 602, ICPL 84023, Pusa 992, Bahar and ICP 8863. These varieties /lines are of intermediate growth habit and many of them are indeterminate. These varieties/lines, as evident from pedigree, have been developed through selection from land races of northern India and south-western India. Potential of most of these germplasms has been identified in ICRISAT. For e.g. ICP 3337 is the ICRISAT pigeonpea accession number of P 4768, from which UPAS 120 is selected and four more genotypes in this cluster has been developed from lines identified in ICRISAT.
- Out of the three lines of the sub-group Ib(i), IPA 3-1 and IPA 3-2 are selections from Bahar x ICPL 96058, whereas, ICPL 88039 is a selection from the germplasm lines of ICRISAT. It is evident that, either they are direct selection or selections from the crosses involving germplasm lines supplied from ICRISAT as resistant donor for wilt and sterility mosaic.
- All the three varieties of the sub-group Ib(ii), viz. CO-5, CO-6 and BMSR 853 are being grown in the same agro-climatic and geographical location i.e. southern and south-western part of India. CO5 and CO6 both are mutant varieties and developed from local land race from south India. Moreover, BMSR 853 is also a progeny having local

land race from south western part of India as parent. These varieties possess semi-spreading/ spreading type of plant.

- Amar, KPL 43 and DA-11 consists the sub-group Ib(iii). These three varieties /lines are of long duration and being grown in north east plain zone of India. All the three genotypes possess erect and compact plant types, and also have one common parent Bahar. Both the varieties of sub-group Ib(iv) i.e. ICPL 87119 and Pusa 9 have been developed through hybridization. Both the varieties possess indeterminate growth habit and semi-spreading to compact plant type. These varieties also possess resistance to sterility mosaic disease.
- Cluster II consists of MAL 6, IPA 402, MAL 13 and PDA 92-1.These genotypes belong to long duration maturity group and are being grown in north east plain zone of India. All the four genotypes of this cluster possess spreading or semi- spreading type of plant. MAL 6, MAL 13 and PDA 92-1 possess one common parent from north-eastern part of India (Bahar), whereas the remaining one is a land race which is also from north-eastern part of India.
- Two varieties viz NDA 1 and T-7 could not be included in any cluster and developed from diverse land races of northern India. Both of them are compact and erect in nature and could be used extensively for breeding programmes for their diversified nature from other cultivars and high yield potential.

Identification of genotypes

- The creation of basic data set using RAPD as a marker system has been done. Moreover, 42 unique products across cultivars as detected by the RAPD analysis (Table 2), which could not only be used as ready reference for varietal identification but also, could be converted into CAPS or SCAR marker for varietal confirmatory tests.

CHICKPEA

Background

Chickpea *(Cicer arietinum* L) or gram, one of the first grain legumes to be domesticated by humans (Vander Maesen 1972) is the third most important pulse crop in the world. In India, it is the premier pulse crop grown largely under rainfed conditions. Major production of chickpea comes from central and northern India. The major chickpea growing states are Madhya Pradesh, Rajasthan, Uttar Pradesh, Maharastra, Gujrat, Andhra Pradesh, and Karnataka. Chickpea occupies one third of the total pulse area (7.2 million hectares) and contributes 42.2% to the total pulses basket of the country,

Assessment of genetic diversity is important step in plant breeding. Molecular markers are superior to morphological or biochemical markers for the diversity analysis since they are independent of the environment and genotype x environment interaction. They are also available in abundance in any genome. In recent years PCR based markers like RAPD and SSR have been very popular for molecular diversity analysis.

Seed protein electrophoresis (Ladizinsky and Adler 1975) and isozyme analysis have been used to establish genetic relationships among *Cicer* species. Those studies revealed minimal intra-specific polymorphism, particularly within *C. arietinum*. Recently, 'Random Amplified Polymorphic DNA (RAPD) analyses have been applied to study genetic relationships among nine annual *Cicer* species (Ahmad 1999). It was shown that RAPD markers could be useful tool for studies of phylogenetic relationships within *Cicer*. Other DNA markers such as ISSRsl microsatellite based, RFLPs or STMSs have been used to estimate variability within *C. arietinum*. On the whole, genetic relationship within *Cicer* spp. based on molecular data is in agreement with other types of analyses used to determine relationships between species. *viz*., karyotype analysis and crossability studies.

Molecular Diversity among the chickpea genotypes

Seventy-five accessions belonging to 14 species of the genus *Cicer* were analyzed with PCR based molecular markers. More than one accession per species was analyzed in most of the wild species where as within *C. arietinum*, 26 accessions including *kabuli* and *desi* types, were taken. RAPD analyses using 12 primers gave 234 polymorphic fragments. Variability within species was detected. A dendrogram based on the Jaccard similarity index showed that the distribution pattern of variability between species was related to both growth habit and geographical origin. Their study suggested the possibility of gene flow between species. Cluster analysis for cultivated chickpea differentiated *Kabuli* and *Desi* types but did not detect a clear relationship between groups and the geographical origin of the accessions (Ahmad, 1999).

Singh *et al.* (2002) analyzed genetic diversity in *Cicer arietinum* L. using Random amplified polymorphic DNA markers to assess genetic diversity in 23 Chickpea genotypes. Forty of the 100 random primers screened revealed polymorphism among the genotypes. Most of the primers revealed single polymorphic band, and only 14.12% of the products were polymorphic. Estimates of genetic similarity based on Jaccard's coefficient ranged from 0.92 to 0.99, indicating narrow genetic variability among the genotypes based on RAPD markers. Twenty three chickpea genotypes formed two major clusters in the dendrogram. The low RAPD polymorphism among chickpea genotypes suggests that higher number of polymorphic primers need to be analyzed to determine genetic relationships. It was observed that RAPD analysis employing 30 polymorphic primers could provide better estimates of genetic relationships in chickpea. In the present study we used more than 40 polymorphic RAPD primers to analyse genetic diversity in 68 varieties of chickpea.

Material

Sixty-eight varieties of chickpea released in India (Table 4) were collected and space planted (60 cm x 30 cm, row to row and plant to plant, respectively). Those cultivars have wide coverage of area in India. The morphological characters were recorded for the entire growing season to weed out the off types. The morphological characters include plant type, growth habit, seed size and colour, height and other related characters. Young leaves were collected from the plants for the DNA extraction.

Table 4. List of chickpea cultivars and their parentage used in diversity analysis

Sl. No.	Genotypes	Parentage	Salient features
1.	GPF 2 (GF 89-36)	GL 769 x H75-35	Medium tall plant, semi-erect, 15g/ 100 seeds, maturity 155-160 days, tolerant to *Ascochyta* blight
2.	PBG 1	GG578 XNEC 206	Tall and erect, small and brown seeds, 14g/100 seeds, maturity 155-160 days, tolerant to *Ascochyta* blight
3.	ICCV 2(K) (Sweta)	K850 x GW 512 x P 48	Semi-spreading plants, white bold seeds. medium tall, maturity 80-90 days, resistant to wilt, tolerant to BGM
4.	JG 315	Selection from WR 315	Erect, seeds brown and medium bold, 14g seeds/100 seeds, maturity 125 days, resistant to wilt
5.	B 108	IP 58 X C2134	Medium tall, semi-erect, maturity 130 days, 15g/100 seeds

6.	PDG 3	GL 769 x GNG 146	Semi-spreading, maturity 160 days, small seeds, 12g/100 seeds
7.	ICCV 10	P1231 X P 1265	Semi-erect, 19.6 g/100 seeds, maturity 95-100 days, resistant to wilt and dry root rot
8.	Vijay (Phule G 81-1-1)	P127 x Annegiri 1	Medium tall and semi-erect, small brown seeds, maturity 105-110 days, resistant to wilt
9.	PDG 4	GL 769 x Flip 85-5C	Semi-erect, small seeded, 21.9g/100 seeds, maturity 140-145 days
10.	BGM 408	Mutant of G130	Semi-spreading, 11.8g/100 seeds, maturity 155 days
11.	Annergiri 1	Local selection from germplasm	Semi-spreading, seeds are yellowish brown and medium bold, 20g/100 seeds, early maturing, duration 100 days
12.	GNG 663 (Vardan)	GNG 16 X GNG 146	Semi-erect plant, medium dark brown Seeds, 13g/100 seeds, maturity 150 days, resistant to wilt
13.	BG 1053 (K) (Chamatkar)	ICCV 3 x FLIP 88-20	Plant are tall and semi-erect white and bold seeds, 21.6g/100 seeds, duration 145-150 days
14.	KPG 59 (Udai)	Radhey x K468	Medium tall and semi-erect, small seeded, 16.3g/100 seeds, maturity 135-140 days, tolerant to wilt, root rot and stunt
15.	KWR 108	Selection from germplasm P108	Medium tall and semi-erect, seeds are brown and small, 18.4g/100 seeds, maturity 135-140 days,
16.	Dahod Yellow	Selection from local germplasm	Medium tall, seeds are yellowish brown and medium in size, 15g/100 seeds, maturity 135-150 days
17.	Pusa 240	H208 x P556	Semi-erect, 22.2g/100 seeds, maturity 135-150 days
18.	BG 267 (K)	US 613 x BEG 482	Plants are semi-erect and tall, seeds are salmon white and bold, 20g/100 seeds, maturity 140-150 days
19.	DCP 92-3	Selection from local germplasm	Semi-erect, medium tall, seeds are yellowish brown and medium bold, 17g/100 seeds, maturity 150 days

20.	Phule G-12	GW 517 x Ceylon 2	Semi-erect, small seeded, 16.9 g/100 seeds, maturity 95-100 days, resistant to wilt and tolerant to pod borer
21.	HK 94-134	H82-2 x E100ym x Bhima	Semi-erect, small seeded, 23.4g/100 seeds, maturity 140-145 days
22.	Pant G 114	G 130 x 1540	Semi-erect, small seeded, 15.3g/100 seeds, duration 140-145 days
23.	JG-74	A composite from genetic stock	Seed are small with coarse seed coat (puckered), 18.3g/100 seeds, wilt resistant
24.	Vishal (Phule G 8720)	K 850 x ICCL 80097	Medium tall and semi erect, 23.6g/100 seeds, duration 112 days
25.	Virat	ICC 7676 x ICCC 32 x ICCC-49 x Flip-82-1C	Semi-erect, bold seeded, 35g/100 seeds, 140-145 days
26.	SAKI 9516	ICCC42 x ICCV 10	Medium seed size, resistant to wilt, 110-140 days
27.	K850	Banda local x Etah bold	Medium tall, semi-erect, seeds are reddish brown, round, smooth and bold, 25.2 g/100 seeds, duration 145-150 days
28.	Sadabahar	Hima x L245	Green seeded, duration 145-150 days, tolerant to wilt
29.	ICCV-37 (K) Kranti	P481 X JG 62 X P 1630	Semi-erect, dwarf plant, light green and narrow leaves, brown and small seeds, 22.5g/100 seeds, duration 105-110 days
30.	BG 212	P340 x G130	Semi-spreading, dark green leaves, light brown oval shaped small seeds, 10.2 g/100 seeds, tolerant to wilt, maturity 135-145 days
31.	RS 10		Semi-spreading, small seeded, 12.7 g/100 seeds
32.	BG 362	BG 303 x P179	Medium tall, semi spreading, medium seeds, 24.3 g/100 seeds, tolerant to wilt
33.	Pusa 209	P827 x C235	Dark green leaves, light brown seed, duration 140-145 days
34.	BG 256	JG 62 x 850-3/27 x L550 x H208	Semi-spreading, medium tall, light brown bold seeds, 24.6g/100 seeds, tolerant to wilt

35.	Chaffa	Selection from Niphad (MS)	Semi-spreading, seeds are yellowish brown and medium bold, 16.3g/100 seeds, duration 105-110 days
36.	RSG 888	RSG 44 x E 100ym	Semi-spreading, small seeded, 14.2g/100 seeds, duration 140 days, twin podded, tolerant to dry root rot and drought
37.	C235	IP 58 x C1234	Medium tall, semi-erect, seeds are small, round wrinkled and brownish yellow, 13.8g/100 seeds, duration 160-170 days, tolerant to *Aschochyta* blight, widely adopted, suitable for rainfed areas
38.	BG 329	BG 203 x P176	Semi-erect, seeds are brown and bold, 16.8g/100 seeds
39.	RSG 2 (Kiran)	A mutant of RS 10	Semi-erect, small seeded, 14.5g/100 seeds, duration 130-135 days
40.	BGM 413	Mutant of G 130	Semi-erect, small seeded, 12g/100 seeds, duration 135-145 days
41.	GCP 101 (Gujrat Gram 1)	GCP 2x ICCV 2	Medium tall and semi-erect, medium bold and dark brown seeds, 20.2g/100 seeds, duration 115-120 days, resistant to wilt
42.	HC-1 (Hare Chole 1)	526 x GG Bijapur	Dwarf plant with long fruiting branch, erect, small and green seeds, 15.4g/100 seeds, duration 170-175 days
43.	BG 391	ICC 3935 x Pusa 256	Medium tall, semi-erect, seeds are bold and dark brown, 21.6g/100 seeds, maturity 110-120 days, moderately resistant to wilt
44.	BGD-72 (Pragati)	Pusa 256 x E100 Ym x Pusa 256	Semi-erect, tall, medium bold seeds, duration 115-120 days, tolerant to wilt
45.	RS-11	Selection from local germ plasm	Semi-erect, 22.6g/100 seeds, duration 135-145 days
46.	Phule G-5 (Vishwas)	B110 x N31	Tall and semi-erect, smooth and round seed, 29g/100 seeds, maturity 110-115 days
47.	RSG –44	JG 62 x F496	Medium tall and semi erect, small, smooth and round seeds, 15g/100 seeds, duration 110-120 days

48.	GNG-146	Selection from unidentified material,	Semierect, purple pigmentation on primary branches, purple flower, seeds grayish yellow and small, 13.6g/100 seeds, duration 135-160 days, resistant to wilt
49.	BG –261(K)	P 327 x P 9847	Erect, small seeded, 14.4g/100 seeds, duration 140-150 days
50.	BG –1003 (K)	Mutant of L532	Semi-erect, white bold seeded, 27.5g/100 seeds, duration 130-140 days, tolerant to wilt
51.	Radhey	197 x 76	Tall plants, semi-erect, seeds are light brown and bold, duration 150-155 days
52.	Vaibhav	Selection from GPICCV 91106	Tall, semi-erect, seeds are small, light brown and bold, 26.3g/100 seeds, duration 120-130 days
53.	RAU –52	ST4 x RS 10	Semi-spreading, deep green, deep brown and small seeds, 12.6g/100 seeds, duration 140-155 days
54.	JG-11	Phule G5 x Narsinghpur x ICCC37	Semi-spreading, bold seeded, 22g/100 seeds, resistant to wilt
55.	GCP 105 (Gujrat Gram 4)	ICCL 84224 x Annegiri-1	Medium tall and semi-erect, green, smooth, rounded and small seeded, 20.5g/100 seeds, duration 120-130 days
56.	Avrodhi	T 3 x K315	Semi-spreading and tall, brown bold seeded, 30.4g/100 seeds, duration 145-160 days, resistant to wilt
57.	L550 (K)	Pb 7 x Rabat	Medium tall and bushy, seeds are small and creamy white, 19.5g/100 seeds, duration 155-160 days
58.	BG 372	P 1231 x P1265	Semi-spreading, small seeded, 14g/100 seeds, moderately resistant to wilt, blight and dry root rot
59.	CSG 8962	Selection from GPF 7035	Medium tall, semi-erect, small brown seeded, 17.9g/100 seeds, tolerant to mild salinity, duration 150-160 days
60.	PBG –5	BG 257 x E100ym	Semi-erect, small brown seeded, 16.8g/100 seeds, duration 160-165 days, tolerant to *Ascochyta* blight

61.	GNG –469 (Samrat)	Annegiri-1 x H75-35	Erect, tall, seeds are brown and bold, 22.6g/100 seeds, duration 145-150 days
62.	L551(K)	ICC 32 x ICCX 780581 BH 10H-BH	Medium tall, bushy, medium bold seeds, 20g/100 seeds, duration 155-160 days
63.	JG130	Phule G5 x Narsinghpur Bold x JG74	Medium tall, brown and bold seeded, 23.9g/100 seeds, duration 135 days
64.	GL 769	H233 x L168	Tall, erect with dark green narrow leaves, small brownish yellow seeds, 14g/100 seeds, duration 160-170 days
65.	GCP-105 (Gujrat Gram 4)	ICCL 84224 x Annegiri-1	Medium tall and semi-erect, dark brown with bold seed, 19.6g/100 seeds, duration 130-145 days
66.	PUSA 244	(850-3/27 x P922) x P9847	Dark green leaves and light brown seeds, 22.5g/100 seeds, duration 110-120 days
67.	KAK –2 (K)	ICCV-2 x Surutato 77 x (ICC 7344-1 CC x 870026-PB-PB-14P-BP-62AK-7AK-BAK)	Medium tall, semi-spreading, bushy, bold seeded, 38g/100 seeds, duration 90-115 days
68	JKG-1	Selection from germplasm	Semi-spreading, bold seeded *kabuli*, early flowering, 35g/100 seeds, duration 125-130 days, wilt resistant

Methodology

General reaction conditions

PCR amplification was performed in 200 μl thin-wall tubes using a MJ Research PTC-200 thermocycler. Reaction solutions (10 μl) contained the 25 ng of genomic DNA template, 0.6 unit of *Taq* DNA polymerase (Bangalore Genei), 200 μM each of dATP, dCTP, dGTP and dTTP (Bangalore Genei), 1.0 μM decamer primer. The PCR buffer consisted of 10 mM Tris-HCl/50 mM KCl/0.1 mg/ml gelatin, pH 8.3 (Bangalore Genei). Cycle conditions were, initial denaturation step at 94^{0}C for 3 min followed by 42 cycles of 94^{0}C for 1 min, 37^{0}C for 1 min and 72^{0}C for 2 min with a final extension at 72^{0}C for 7 min.

Diversity analysis

Amplification products were resolved by electrophoresis on 1.2% agarose gels for RAPD and 2% agarose gel for SSR and visualized with UV illumination.

Table 5a: List of RAPD markers used and the presence of polymorphism for different primers

Primer	Sequence	Maximum no. of bands	Polymorphic(P)/ Non polymorphic (NP)/ Not Amplified (NA)
OPAC –4	ACGGGACCTG	4	NP
OPAC-6	CCAGAACGGA	1	NP
OPAC-7	GTGGCCGATG	3	NP
OPAC-8	TTTGGGTGCC	7	P
OPAC -9	AGAGCGTACC	NA	NA
OPAC-10	AGCAGCGAGG	7	NP
OPAC-11	CCTGGGTCAG	6	P
OPAC-12	GGCGAGTGTG	4	P
OPAC-13	GACCCGATTG	NA	NA
OPAC-3	CACTGGCCCA	5	P
OPAC-18	TTGGGGGAGA	1	NP
OPAC-20	ACGGAAGTGG	1	NP
OPBC-1	CCTTCGGCTC	NA	NA
OPBC-2	ACAGTAGCGG	4	P
OPBC-3	GGCTTGACCT	4	P
OPBC-6	GAAGGCGAGA	4	P
OPBC-7	TGTGCCTGAC	5	P
OPBC-8	GGTCTTCCCT	3	P
OPBC-10	AACGTCGAGG	3	P
OPBC-11	TTTTGCCCCC	3	P
OPBC-12	CCTCCACCAG	5	P

OPBC-13	CCTGGCACAG	4	P
OPBC-14	GGTCCGACGA	4	NP
OPBC-15	CCAGACTCCA	5	P
OPBC-16	CTGGTGCTCA	5	P
OPBC-17	CCGTTAGTCC	6	NP
OPBC-18	GTGAAGGAGG	4	P
OPBC-19	ACAAGCGCGA	1	NP
OPBE-1	CACTCCTGGT	3	P
OPBE-2	ACGCCTGTAG	3	P
OPBE-3	TGGACTCGGT	6	P
OPBE-4	CCCAAGCGAA	7	NP
OPBE-5	GGAACGCTAC	9	P
OPBE-6	CAGCGGGTCA	7	P
OPBE-7	CCGTCCTATG	4	P
OPBE-8	GGGAAGCGTC	9	P
OPBE-9	CCCGCTTTCC	4	P
OPBE-10	AAGCGGCCCT	3	P
OPBE-11	GTCCTGCTGT	4	P
OPBE-14	CTTTGCGCAC	3	NP
OPBE-15	TTCGGCGATG	3	P
OPBE-16	CTCCACGACT	5	P
OPBE-17	GGGAAAAGCC	5	P
	Total		174

Table 5b: List of RAPD markers used and the presence of polymorphism for different primers

S.No.	PRIMER	No. of Polymorphic Bands	No. of Monomorphic Bands	Total No. of Bands
1	OPAZ-1	8	1	9
2	OPAZ-3	2	2	4
3	OPAZ-4	3	1	4
4	OPAZ-5	3	2	5
5	OPAZ7	2	4	6
6	OPAZ-8	0	1	1
7	OPAZ-9	3	2	5
8	OPAZ10	4	0	4
9	OPAZ-11	6	0	6
10	OPAZ12	4	0	4
11	OPAZ-13	13	0	13
12	OPAZ-14	7	1	8
13	OPAZ-16	6	0	6
14	OPAZ-18	0	1	1
15	OPAZ-19	0	1	1
16	OPAZ-20	0	1	1
17	OPAM-1	3	2	5
18	OPAM-2	9	0	9
19	OPAM-3	3	0	3
20	OPAM-4	4	1	5
21	OPAM-5	4	1	5
22	OPAM-6	1	1	2
23	OPAM-7	3	3	6
24	OPAM-8	0	2	2
25	OPAM-9	4	2	6
26	OPAM-10	3	1	4

(Contd .....)

27	OPAM-11	9	0	9
28	OPAM-12	0	1	1
29	OPAM-13	5	5	10
30	OPAM-14	5	2	7
31	OPAM-16	6	3	9
32	OPAM-17	0	3	3
33	OPAM-19	13	0	13
34	OPAM-20	1	3	4
35	OPAC-1	5	0	5
36	OPAC-2	6	3	9
37	OPAC-3	1	2	3
38	OPAC-4	1	3	4
39	OPAC-5	4	1	5
40	OPAC-6	0	3	3
41	OPAC-7	8	0	8
42	OPAC9	3	1	4
43	OPAC-10	0	2	2
44	OPAC-11	O	1	1
45	OPAC-12	1	2	3
46	OPAC-13	3	0	3
47	OPAC-14	4	1	5
48	OPAC-15	1	1	2
49	OPAC-18	5	0	5
50	OPAC-20	3	1	4
i	Total	179	68	247
ii	Total %	72.4	27.5	
iii	Mean	3.58	1.36	4.94
iv	Mean %	72.4	27.5	

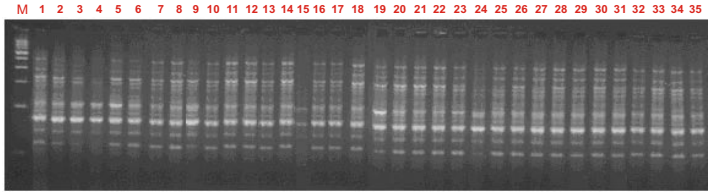


Fig 4 a.

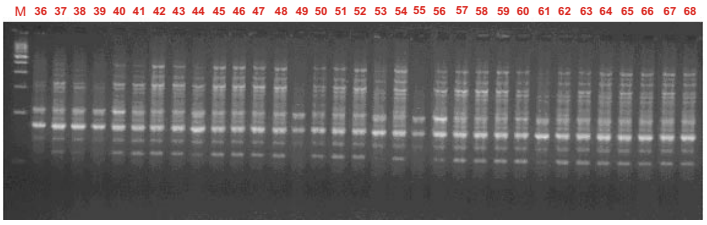


Fig 4b

Fig. 4: Lane M: 500 bp DNA ladder, lane 1-68 are the chickpea cultivars (as in M & M) amplified with RAPD marker, OPAM-14

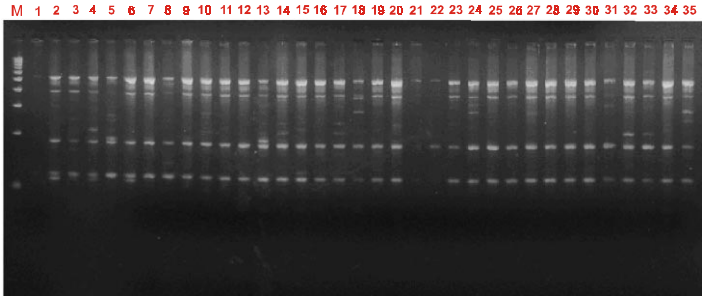


Fig 5a

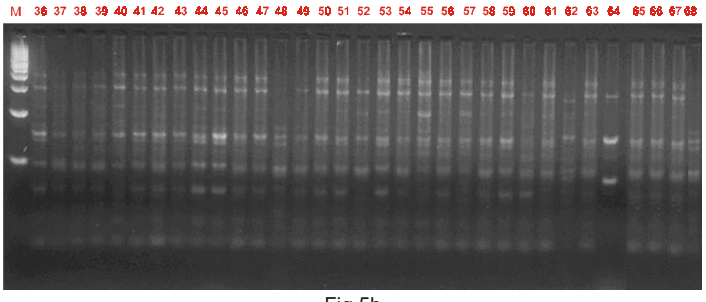


Fig 5b

Fig. 5: Lane M: 500 bp DNA ladder, lane 1-68 are the chickpea cultivars (as in M & M) amplified with RAPD marker, OPAC-1

Statistical analysis

The banding patterns obtained with the DNA amplified with RAPDs were analysed to estimate genetic relationships among the 68 chickpea cultivars. Amplified samples were scored for the presence (1) or absence (0) of homologous bands to create a binary matrix of the different RAPD, genotyped by using Alpha DigiDoc software. A cluster analysis based on the similarity matrix was performed using the unweighted pair group method with arithmetical averages (UPGMA). NTSYS–ps version 2.0 software was used for cluster analysis and then the dendrogram was prepared.

Significant Findings:

- A total of 50 RAPD primers were used for the DNA fingerprinting of the chickpea cultivars. Out of 50 primers, 20% (10) did not give any polymorphism. Rest 80% (40) primers gave at least one polymorphic band.

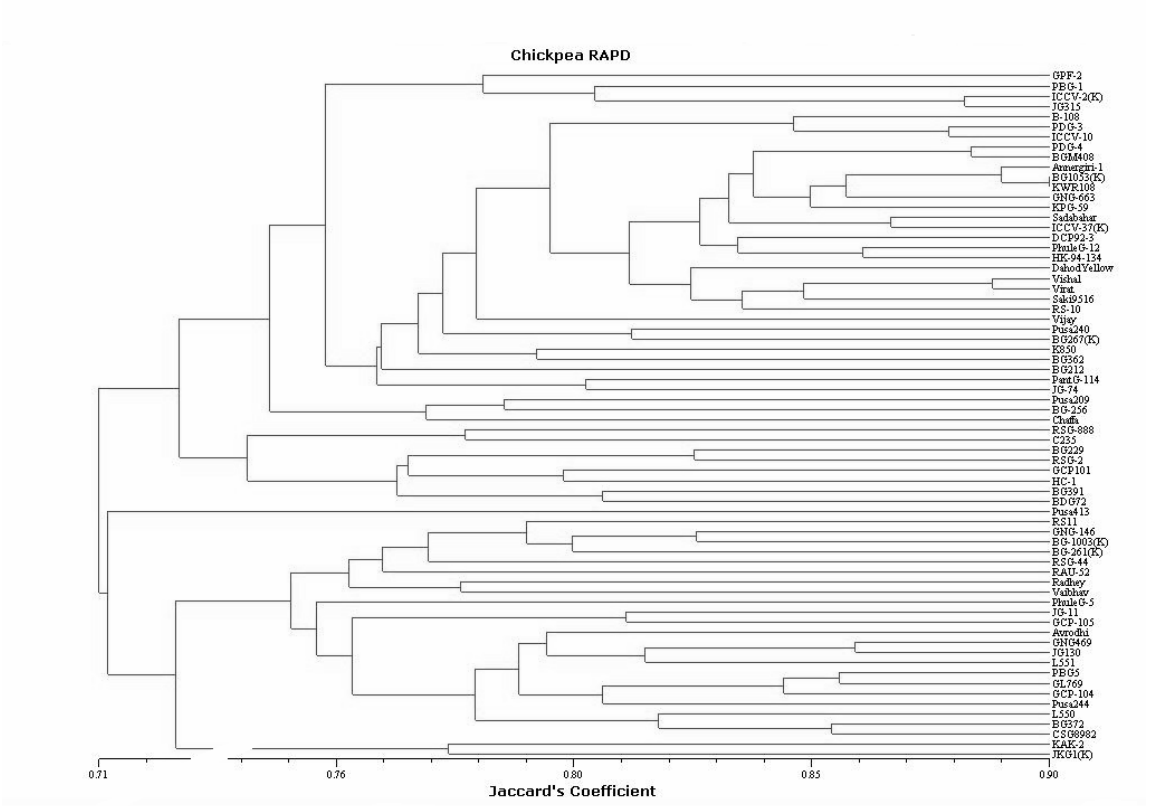


Fig 6: Dendrogram derived from random amplified polymorphic DNA analysis of 68 chickpea varieties

- A total of 247 bands were generated by 50 RAPD markers. Out of which 179 (72.4%) were polymorphic where as 68 (27.5%) were non-polymorphic. A mean of 4.94 markers were observed per primer.
- The estimates of genetic similarity based on Jaccard's coefficient ranged from 0.71 to 0.90.
- At the similarity level of 29%, there are two main clusters of cultivars where as BGM 413 makes a different cluster, indicating it is quite different to rest of the cultivars.
- The cultivars BG 1063 and KWR 108 are very closely placed in the dendrogram. BG 1063 is *kabuli* type where as KWR 108 is *desi* type. Hence, Desi and Kabuli types did not differentiate into two groups using RAPD as well as SSR markers (Fig. 6).
- The range of estimates of genetic similarity based on Jaccard coefficient (0.71 to 0.90) is not very high.

MUNGBEAN

Background

Mungbean [*Vigna radiata* (L) Wilczek 2n = 2x = 22] is one of the most important edible food legume of Asia. In India, mungbean occupies an area of 3.34 million ha and produces about 1.06 million tones with productivity of 317kg/ha (FAO, 2005). In India and some south Asian countries, it contributes significant dietary protein supply in predominantly cereal rich diets. Mungbean suffers heavy losses due to biotic and abiotic stresses and consequently historically the productivity has remained low and unstable. Despite major efforts through conventional breeding, the gap between potential yield and realized yield still remains large. Hence the challenge now is to improve the efficiency of mungbean breeding using the latest molecular marker which have shown great promises in the crop species.

The genetic diversity within released cultivars of Indian mungbean has been rather sporadic and commonly used parents in breeding programs have missed out from these studies. It has been described that diversity of Indian mungbean cultivars is narrow. Lakhanpaul *et al.* (2000) studied the diversity among 32 mungbean cultivars from India using RAPD markers. Singh (2003) used ISSR marker to access genetic diversity to mungbean genotypes. To have an assessment of genetic diversity and relationship among the cultivars, Bhat *et al.* (2005) used AFLP marker system. In these studies close relationship between the cultivars was explained on the basis of high degree of commonness between their pedigrees. These studies also revealed a narrow genetic base of Indian mungbean. Genetic diversity among mungbean germplasm was estimated using RAPD technique by Santalla *et al.* (1998). This study provided evidence in support of the separation of Asiatic and African members of the genus *Vigna*. It justified the specific distinction between *V.radiata* and *V.mungo* as separate but closely related species.

Relative efficiency of different primer lengths in revealing polymorphism in 46 mungbean genotypes was studied by Saini *et al.* (2004). Long primers of 18-22 bases and 10 bases primers were evaluated for total number of discrete and detectable amplified fragments and polymorphic bands. The results showed that for a large germplasm collection, long primers are more useful in revealing diversity. Bhat *et al.* (2005) studied relative efficiency of AFLP primers having 3 or 2 selective nucleotides for detecting polymorphism. Higher percent of polymorphism was obtained with +3 then with +2 though the number of amplification products were much higher with +2 primers.

The assessment of genetic diversity among 24 released cultivars of mungbean have been done using 10 base long random primers.

Material

The plant materials used for this study included 24 released cultivars of mungbean popularly grown in different regions of India (Table 6). Seeds of these varieties were collected from germplasm section of IIPR, Kanpur and were grown under controlled conditions. The off-type plants were removed and pure seeds true to each cultivar were harvested.

Table 6: Description of mungbean varieties used in diversity analysis

S. No.	Varieties	Pedigree	Year of Release	Days of Maturity(d)	Special Feature
1.	BDYR 2	AVRDC Line	-	60-65	Bold Seeded
2.	K 851	4453 X 3T44	1982	60-65	Shining green smooth egg shape and bold seed, spreading.
3.	PM 4	Type 44x UPU 2	1992	65-67	Resistant to YMV
4.	Pusa 105	Taiwan 1 x ML 6 x EGMG 16 x ML3	1983	65-70	Erect, Determinate, PM and YMV (R)
5.	MUM 2	Mutant of K 851	1992	60-70	Tolerant to MYMV, medium size seed
6.	Pusa Vishal	Selection From NM 92	2000	60-65	Resistant to YMV
7.	M L 729	Donors	1995	65-67	Tolerant to powdery mildew
8.	Pusa 9072	Pusa 106x 10-215	1995	65-70	Tolerant to Powdery Mildew
9.	M L 843	Donors	-	-	-
10.	ML 682	Donors	-	-	-
11.	NM 1	G 65x UPM 79-34	1992	60-70	Resistant to YMV
12.	I P M 02-03	Pusa Vishal x IPM 99-125	-	-	-
13.	Pant Mung 2	Mutant of ML 26	1984	60-70	Shining green seed
14.	Pant Mung 3	LM 294-1 x L 80	1985	75-85	Seed dull green, erect PM(MR), YMV (R)
15.	Ganga 8	K 851 x Pusa 105	2001	72	Moderately resistant to YMV, CLS etc.
16.	PDM 11	Selection from LM 595	1987	70-75	Shining green seed erect, bushy, Suitable for spring season
17.	Pratap	Selection from Shillong local	1993	60-70	Suitable for early and late situations
18.	TAP 7	Mutant of S-8	1982	62-65	Small shining green seed, erect, determinate
19.	Asha	K 851 x L 24-2	1993	75-80	MYMV (MR)

20.	GM 3	PIMS 4 X 11-99	1995	-	-
21.	SML 134	V 2164 x ML 258	1996	68	For spring/ Summer
22.	TARM 1	RUM 5 x TPM-1	1996	80-85	Small green seed, resistant to mildew and tolerant to MYMV
23.	ML 5	No. 54 x Hyd 45	1976	80-85	Erect, Determinate, CLS & YMV (MR)
24.	AKM 8803	PIM 53 x MH-1	1992	65-70	MYMV(MR)

Methodology

Seeds of each variety (10-12 seeds) were germinated in paper towels soaked with sterile water. Seeds were grown in etiolated condition to reduce any chloroplast DNA contamination. After 7 days, the seedlings were harvested and 1g of each sample was used for isolation of genomic DNA using a modified method of George *et al*. (1997). The samples were crushed in 15 ml extraction buffer (containing 3M CH_3COONa, 4M NaCl, 0.5 M EDTA pH 8.0, 1M Tris pH 8.0, 2% PVP, 1.4%SDS), and subjected for 1 hour incubation at 65°C on water bath. Three ml of CH_3COONH_4 (pH4.8) was added and then sample was centrifuged at 12,000rpm in Sorvall SS34 rotor for 10 minutes. Supernatant was collected in new tubes to which 0 .6 volume isopropanol was added to precipitate the DNA and kept for 2 hours. DNA was pelleted by centrifuging the tubes at 12,000 rpm for 10 minutes. The supernatant was discarded and pellet suspended in 1 ml $T_{10}E_1$ buffer (10mM Tris-HCl and 1mM EDTA). After that samples were extracted with an equal volume of phenol/ chloroform/ isoamyl alcohol (25:24:1) mixture followed by equal volume of choloform/isoamyalcohol (24:1). The suspension was mixed well and centrifuged for 5 minutes at 12,000 rpm and supernatant was collected. The DNA was precipitated with 100% cold Ethanol through 20 minutes centrifugation at 12,000 rpm. The DNA was washed with 70% ethanol through 5 minutes centrifugation at 12,000 rpm and suspended in $T_{10}E_1$ buffer.

For quantification of the isolated DNA, known concentration of uncut λ phage DNA was electrophoresed along with sample DNA at 70V for 2 hours using 1X TBE buffer in 0.8% agarose gel. This was also confirmed by a DNA fluorometer reading (Hoefer Scientific,CA,USA) using calf thymus DNA as a blank standard and Hoechest 33258 DNA intercalating dye.

RAPD Analysis

Sixty primers belonging to OPD, OPX and OPI series of random decamer primers from Operon Technologies, USA were surveyed for PCR amplification in the selected 24 mungbean cultivars. PCR conditions were standardized using varying concentrations of primers and template DNA. After standardization, the reaction were carried out in 25µl volume and

contained 2.5 µl of 10X Taq buffer, 2.5 µl of 2mM dNTP mix, 30nM primer, 1 unit of Taq polymerase and 25ng of template DNA. The reaction mixtures were prepared in a laminar flow cabinet to avoid any airborne microorganism contamination. Because, the PCR reaction involved use of multiple cultivars, a master mix was prepared first, containing water, buffer, dNTPs, primers and Taq polymerase in a single tube and aliquoted to tubes containing template DNA from individual cultivars. We used thin walled, flat cap PCR tubes from Axygen for all PCR reactions. The thermal cycling program was carried out in a PTC Thermal cycler (MJ Research/Biorad). The PCR program had an initial denaturation step at 94°C for 4 min, followed by 44 cycle of 94°C for 1 min, 37°C for 1 min, and 72°C for 2 min. A final extension step was given at 72°C for 7 min.

Agarose gel electrophoresis

The amplified products were electrophoresed at 75V in 1.2% Agarose gel in 1X TBE buffer for 3 hours .The DNA bands were visualized by staining gel in 1% ethidium bromide solution and photographed under UV light using a Alpha image Digidoc gel documentation system. A100bp DNA ladder (MBI Fermentas) was used as a molecular weight marker for determining the molecular weight of the amplified products.

Scoring and analysis of data

Digitized gel photograph of RAPD results were analyzed using NTSYS PC Ver.2.0 numerical software package. Data was recorded as 1 (presence) or 0 (absence). The bands which were very faint were not considered for scoring. For each primer, PCR reactions were repeated two times and for scoring purpose only reproducible bands were considered. The primers which did not produce amplification were repeated at least three times before discarding them. The pair wise similarity between isolates and polymorphic bands were calculated using Jaccard's coefficient (1980). The UPGMA methods was used to generate a dendogram using matrix of similarity coefficient. WinBoot software program was used to perform bootstrap analysis of binary data to determine the confidence limits of UPGMA based dendograms.

Significant Findings

RAPD Analysis

- A total of sixty RAPD primers from OPERON were screened to assess the diversity in the 24 mungbean varieties. Out of sixty primers tested, thirty three of them generated reproducible RAPD patterns. Thirty-six primers amplified a total of 249 bands. Thus the average number of bands amplified per primer was 7 (Table 7).

- Out of thirty-six primers, twenty-three primers showed more than 80% polymorphism. The total number of polymorphic bands amplified was 224 (90%). Among the responding primers, OPD-7 produced maximum number of bands (17) with 100% polymorphism while primer OPX -5 produced the minimum number of bands (3) with 33% polymorphism. The size of products ranged from 2.6 kb to 0.25 kb. Fig 7 shows the amplification profile generated using primer OPD 07 and OPD 15.

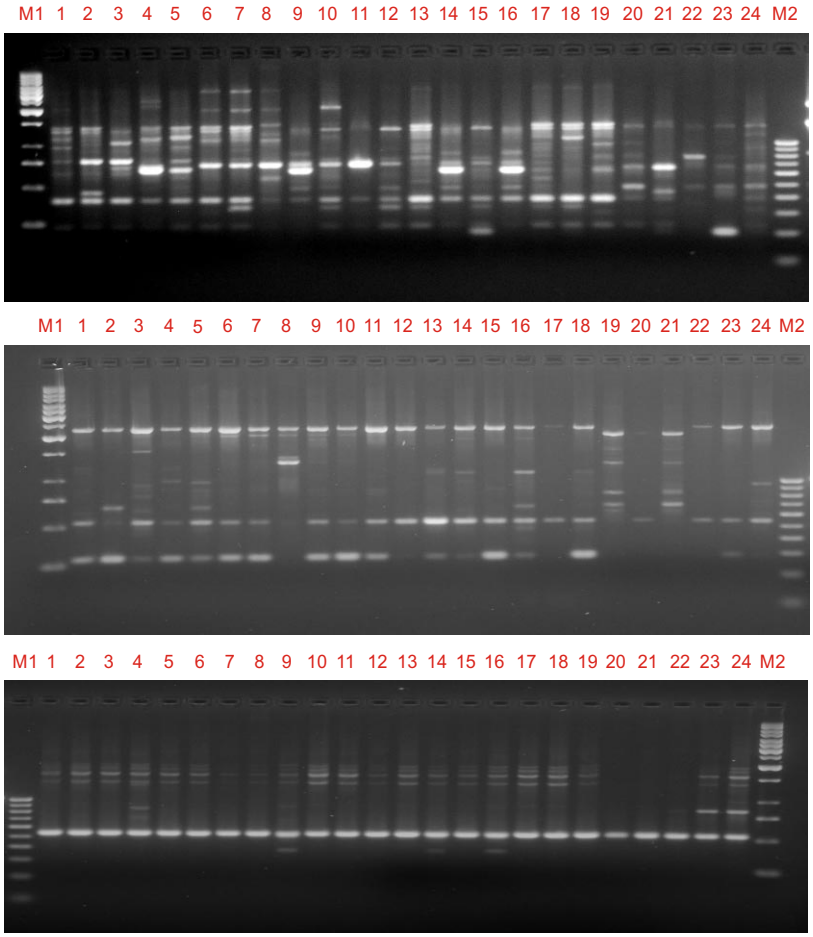


Figure 7: RAPD profile of 24 mungbean varieties using primer (a) OPD 07 (b) OPD 15 (c) OPD Lanes: M1: Marker DNA, 1 kb ladder. Lane M2 100 bp ladder : Marker DNA, Lanes 1-24 : Different varieties of mungbean.1:BDYR2, 2:K851, 3:PM4, 4:Pusa105, 5:MUM2, 6:Pusa Vishal, 7:ML729, 8:Pusa 9972, 9:ML 843, 10:ML 682, 11:NM1, 12:IPM 02-03, 13:Pant Mung2, 14:Pant Mung3, 15:Ganga 8, 16: PDM11, 17: Pratap, 18:TAP7, 19:Asha, 20:GM 3, 21:SML134, 22:TARM1, 23:ML5, 24:AKM 8803.

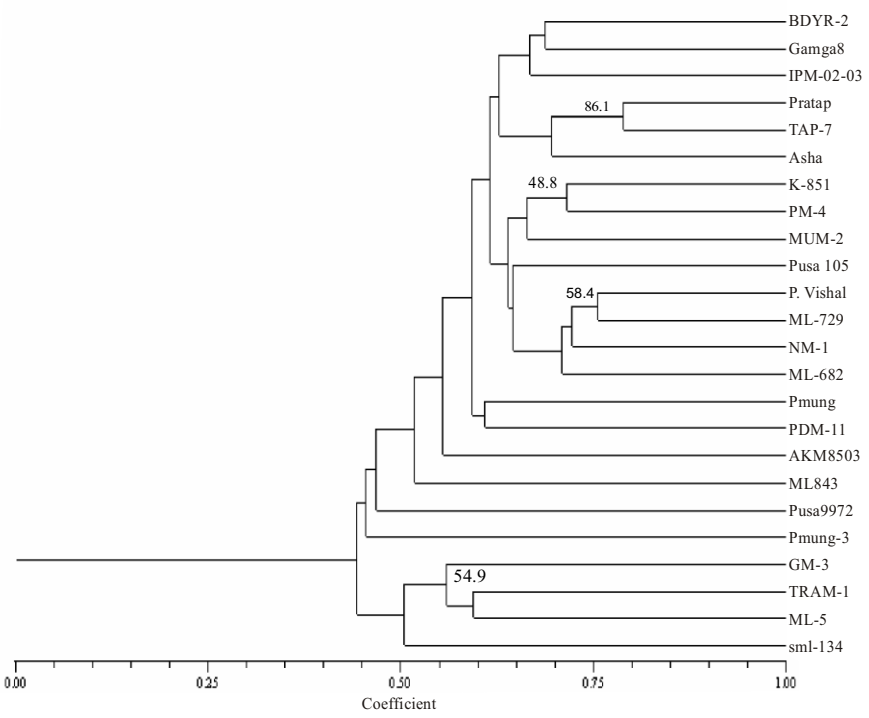


Fig. 8: Dendrogram derived from random amplified polymorphic DNA analysis of 24 mungbean varities

Table 7: Scoring of polymorphism in 24 mungbean varieties using RAPD primers

Primer	Total number of bands	Polymorphic bands	Monomorphic bands	%of Polymorphic bands
OPX-2	7	6	1	85. %
OPX-3	11	10	1	90. %
OPX-4	13	13	0	100%
OPX-5	3	1	2	33. %
OPX -6	8	8	0	100%
OPX-9	9	8	1	88%
OPX-11	9	8	1	88%

OPX-13	6	6	0	100%
OPX-15	7	7	0	100%
OPX-19	13	13	0	100%
OPX-20	6	5	1	88.%
OPD-2	6	4	2	66.%
OPD-5	11	11	0	100%
OPD-7	17	17	0	100%
OPD-8	7	5	2	71.%
OPD-11	11	10	1	90.%
OPD-13	6	5	1	83.%
OPD-18	8	7	1	87.%
OPD-20	14	14	0	100%
OPI-2	5	3	2	60%
OPI-3	4	3	1	75%
OPI-4	8	8	0	100%
OPI-6	6	6	0	100%
OPI-9	2	1	1	50%
OPI-11	4	3	1	75%
OPI-12	7	5	2	71%
OPI-13	7	7	0	100%
OPI-14	7	7	0	100%
OPI-15	5	4	1	80%
OPI-16	3	2	1	66%
OPI-17	5	3	2	60%
OPI-18	7	7	0	100%
OPF-1	7	7	0	100%

- A dendrogram based on UPGMA analysis revealed the genetic diversity among the twenty four varieties of mungbean which ranged from 0.45 to 0.78, but mostly concentrated between 0.60 and 0.75. The RAPD cluster pattern is presented in Fig 8. It showed two main clusters, Cluster I comprising of sixteen varieties in five sub clusters (IA to IE) and cluster II comprises of four varieties. But, four varieties AKM 8803, ML843, Pusa 9972 and Pant Mung took an independent position.
- Sub cluster IA comprised of three varieties for which place of release could not be traced. Sub cluster IB consist of three varieties, one from BARC and one variety released from CCSHAU, Hissar, sub cluster IC also comprise of three varieties K-851 released from CSAUA&T, Kanpur, Pant mung 4 released from Pantnagar, MUM 2 released from Meerut, sub cluster ID consist four verities Pusa Vishal released from IARI, New Delhi and for other three varieties place of release could not be traced, sub cluster IE comprise of two varieties one from IIPR, Kanpur and one from Pantnagar. Cluster II comprise of four varieties in which two were released from PAU, Ludhiana, one from BARC and one from GAU, Gujarat.
- On Bootstrap analysis of the clusters of dendogram, cultivars TAP 7 and Pratap in sub cluster IB shared 86%. The genetic similarity was also highest (78%) among these two cultivars ,while Pusa Vishal and ML 729 in sub cluster ID showed 75% genetic similarity with bootstrap value of 58.4 %. In sub cluster 1C, K-851 and PM 4 were showing a bootstrap value of 48.8% at a genetic diversity of 71%. In Cluster II TRAM-1 and ML 5 share 59% genetic similarity with 54.9% bootstrap value.

Lawn and Ahn (1985) assessed the genetic diversity and phylogenetic relationships in plant genetic resources.Several workers have also reported the usefulness of RAPD (William *et al.* 1990) technique in studying the diversity of crop cultivars. RAPD markers have been used successfully to assess molecular polymorphism in *Vigna* (Kaga *et al*.1996), mungbean (Santalla *et al*.1998, Lakhanpaul 2000), and in cowpea (Mignouna *et al.* 1998).

For studying the genetic diversity in crops, several methods are employed and all the methods have inherent assumption. The use of appropriate method especially in case of RAPD analysis is very important to make genetic variation more definitive. The UPGMA is based on the assumption that mutation rate among different genotypes is constant and this has been widely used for analysis of genetic variation in plants. In our study for analysis of RAPD polymorphism, we have used this tool and tested that the cluster obtained are reproducible. In this study the objective was to examine the relationship among 24 commonly grown mungbean cultivars of India. In recent years a number of studies have been undertaken, the genetic diversity among the selective cultivars was reasonably high. This may be ascribed to study the fact that the parentages of the cultivars were very diverse and no two cultivars shared common parent. Lakhnapual *et al.* (2000) reported that cultivars originating from same area usually cluster together. But we have observed that all the mungbean varieties

released from IARI Pusa, New Delhi fall into different sub clusters in the dendogram. Among these Pusa Vishal is relatively new variety released in 2000. But PUSA 9072 is about a decade old (released 1995) and Pusa105 is almost 20 years old (released in 1987). Consciously or unconsciously artificial selection takes place in seed production programs. In addition to this natural selection and spontaneous mutation may aid in further diversification of varieties. Similarly two varieties released from Pantnagar are more than two decade old and are placed in two clusters. Even on the basis of morphological and agronomical traits, clustering pattern does not necessarily reflect geographical origin. Bisht *et al.* (1998) used 29 traits to group 111 accessions of mungbean. The six clusters thus obtained, had no correlation with geographic diversity. These observations reveal that genotypic diversity among the cultivars may be due to the other factors like selection, mutation etc. For successful exploitation of genetic diversity in a crop species, germplasm and exotic collection should be explored. For improvement of exiting cultivars and development of new cultivars, it is important to use consensus set of core and mini core germplasm collection for diversity analysis which has been shown to represent the entire diversity in the species using morpho - agronomic descriptors.

LENTIL

Background

Lentil is the second most important *rabi* pulses of India. Out of a total area of 73.2 million ha under pulses globally, lentil occupies 4.0 m ha producing 4.03 m tones of grains. In India, it occupies 1.4 m ha and produces 1.0 m tones. The other important lentil growing countries are Turkey, Syria, Pakistan, Spain and Bangladesh. India rank first in the world in respect of production as well as area followed by Turkey. In India, lentil is grown mostly in the states of M.P., U.P., Bihar and West Bengal.

In order to improve the yield potential of a crop, the breeder uses diverse parents to bring the desirable alleles into elite and widely adapted backgrounds. In this process, he/she evaluates the germplasm and releases cultivars under a given environment, where his/her observations are influenced by the environment and genotype x environment interactions. The diversity analysis of the genotypes at the DNA level eliminates these two factors. DNA markers like RFLPs, RAPDs, SSRs, STSs, STMSs, and AFLPs are quite abundant in a genome and are very useful tools to understand the existence of diversity among the genotypes. In the present study we have used RAPD and SSR markers because of their speed and fair amount of reliability.

Both RAPD and SSR markers have been used for the diversity analysis within and amongst the lentil populations. RAPD markers were used to distinguish between six different *Lens* taxa representing cultivated lentil and its wild relatives. RAPD analysis for the relationships between 16 accessions and cultivars of lentil were examined in the Australian lentil-breeding programme (Ford *et al.,* 1997). All lines exhibited polymorphism with a maximum dissimilarity value of 0.36. Analysis of molecular variance of both isoenzyme and RAPD data revealed that between 78% and 99% of the variation was attributable to between-population differences. Similarly, RAPD data for several geographically dispersed accessions/ cultivars of four diploid *Lens* species provided important evidence about the origin of the cultivated lentil and also measured genetic variability in lentil germplasm (Ahmad *et al*. 1996).

Sharma *et al.* (1996) used AFLP and RAPD marker techniques to evaluate and study the diversity and phylogeny of 54 lentil accessions representing six populations of cultivated lentil and its wild relatives. The results of AFLP analysis were compared to results from a previous RAPD analysis of the same material. The two methods provide similar conclusions as far as the phylogeny of *Lens* is concerned. DNA polymorphism were also detected by analysis of variable number of tandem repeats (VNTR), microsatellite and minisatellite DNA sequences, inter-simple sequence repeats (ISSR), and amplified fragment length polymorphism (AFLP) to distinguish between commercial lentil cultivars (*Lens culinaris*) that are closely related or identical judging from pedigree and agronomical and morphological

traits. In a similar study, cultivated lentil and several wild species distributed from the Mediterranean region to western Asia were studied to end persisting uncertainty regarding the phylogeny of the genus by analyzing the variation in the ITS region among species. The results were in congruence with RFLP studies.

Several centres of genetic diversity have been recognized for lentil, in particular Afghanistan, Turkey and Ethiopia. Biosystematic, morphological, geographical and ecological as well as, hybridization, cytogenetic, biochemical, taxonomical, archaeological, and evolutionary studies of the common pea, faba bean (*Vicia faba*), grass pea (*Lathyrus sativus*), lentil (*Lens culinaris*) and chickpea (*Cicer arietinum*) has showed that all have originated in the Fertile Crescent. The wild progenitor can be identified for all species except *V. faba* and considerable divergence has occurred in the other species.

Cluster analysis in lentil showed that the landraces that originated in Iran and Syria, and the breeding lines developed at ICARDA are distinctly different from the lentil accessions that originated in countries at more southerly latitudes (India and Ethiopia). However, out of 40 genotypes, only one line (ILL 6002) was strikingly different from all other test genotypes. This line exhibited significantly superior root and shoot traits and yield, and, therefore, is a valuable germplasm for breeding drought tolerant cultivars.

Material

Thirty-one genotype of lentil including 26 cultivars grown in India were taken for DNA fingerprinting and diversity analysis (Table 8). Those 31 genotypes included two exotic genotypes *viz* Masor and Precoz. Precoz (ILL 4605) from Argentina used in this study, was the first early flowering, macrosperma which were used in lentil crossing programme in 1990. These two genotypes were also used as checks for understanding the diversity among the lentil cultivars.

Table 8: Genotypes of lentil used for the DNA fingerprinting and diversity analysis.

S. No.	Genotype	Parentage	Salient features
1	DPL 15 (Priya)	PL 406 x L 4076	Semi-spreading, dark green foliage, purple flower, saffron cotyledon, 135-140 days duration, bold seeded (2.79 g/100 seeds), resistant to rust
2	K 75 (Malika)	Local selection	Semi-erect, light green foliage, purple flower, saffron cotyledon, bold seeded, (3.0g/100 seeds)
3	LH 84-8 (Sapna)	L 9-12 x JLS -2	Semi-erect, light green foliage, purple flower, saffron cotyledon, matures in 135-140 days, bold seeded (2.7g/100 seeds), tolerant to rust

4	LH 82-6	Pusa 2 x No 4	Semi-erect, dark green foliage, purple flower, saffron cotyledon, medium bold seeded
5	DPL 62 (Sheri)	JLS –1 x LG -171	Semi-erect, dark green foliage, purple flower, saffron cotyledon, less hairy, resistant to rust and wilt, matures in 130-135 days, bold seeded (3.4 g/100 seeds)
6	T-36	Selection at Kanpur	Semi-erect, light green foliage, purple flower, saffron cotyledon, small seeded
7	LL 147	Pl 284-67 x NP21	Semi-erect, light green foliage, purple flower, saffron cotyledon, small seeded, tolerant to rust and wilt
8	L 4147 (Pusa Vaibhav)	(L 3875 x P4) x PKVL 1	Semi-erect, light green foliage, purple flower, saffron cotyledon, small seeded, maturity 134 days, resistant to rust
9	L 4076	PL 234-67 x PL 639	Semi-erect, light green foliage, purple flower, saffron cotyledon, bold seeded (3.1 g/100 seeds), resistant to rust
10	L 9-12	Selection from local	Semi-erect, light green foliage, purple flower, saffron cotyledon, small seeded, wide adaptability, tolerant to rust
11	PL 406	Selection from P-495	Semi-erect, light green foliage, purple flower, saffron cotyledon, small seeded, rust resistant, wilt tolerant
12	PL 639	L 9-12 x T-8	Semi-spreading, light green foliage, purple flower, saffron cotyledon, widely adapted, 135-140 days, small seeded (1.9g/100 seeds), resistant to rust, tolerant to wilt and root diseases
13	PL 4	UPL –175 x (PL 184 x P 288)	Semi-erect, dark green foliage, purple flower, saffron cotyledon, small seeded, (1.9 g/100 seeds), resistant to rust and wilt/ root rot, matures in 135-140 days
14	PL 5	L4163 x LG 171	Semi-erect, dark green foliage, purple flower, yellow cotyledon, medium bold seeded
15	PL 234	Selection from P-230	Semi-erect, light green foliage, purple flower, saffron cotyledon, medium bold seeded, tolerant to rust and wilt
16	L 4603	Precoz x L3991	Semi-erect, light green foliage, purple flower, saffron cotyledon, medium bold seeded
17	IPL 525	PL 639 x Precoz	Semi-spreading, dark green foliage, purple flower, saffron cotyledon, small seeded

18	Sehore 74-3	–	Semi-erect, dark green foliage, purple flower, saffron cotyledon, small seeded
19	PL 77-2	Mutant of B -25	Semi-erect, dark green foliage, purple flower, saffron cotyledon, medium bold seeded
20	Mason	Exotic collection from USA	Semi-erect, light green foliage, purple flower, yellow cotyledon, medium bold seeded
21	DPL 58	PL 639 x Precoz	Semi-erect, dark green foliage, purple flower, yellow cotyledon, bold seeded
22	Precoz	Exotic collection from Argentina	Semi-erect, light green foliage, purple flower, yellow cotyledon, bold seeded
23	B 77 (Asha)	Selection from Jorhat local	Semi-erect, dark green foliage, white flower, saffron cotyledon, small seeded
24	IPL 81 (Noori)	K –75 x PL 639	Semi-erect, dark green foliage, purple flower, saffron cotyledon, tolerant to rust and wilt, matures in 115-120 days, bold seeded (2.7 g/100 seeds)
25	Ranjan	Mutant of B -77	Semi-erect, dark green foliage, white flower, saffron cotyledon, small seeded (1.8 g/100 seeds)
26	WBL 58 (Subrata)	JLS 2 x T -36	Semi-erect, dark green foliage, purple flower, saffron cotyledon, bold seeded, tolerant to rust, matures in 120-125 days
27	VL 1	Selection from local	Semi-spreading, dark green foliage, purple flower, saffron cotyledon, seed coat black, small seeded (1.9g/ 100 seeds), tolerant to wilt and rust
28	JL 1	Selection from local (Almora)	Semi-erect, dark green foliage, purple flower, saffron cotyledon, medium bold seed
29	VL 103	–	Semi-erect, dark green foliage, purple flower, saffron cotyledon, small seeded
30	VL 4	Local selection from Pithoragarh	Semi-erect, dark green foliage, purple flower, saffron cotyledon, small seeded, black seeded, tolerant to rust and wilt
31	NDL 1	Precoz x L 9-12	Semi-erect, dark green foliage, purple flower, saffron cotyledon, bold seeded, resistant to rust and wilt

Methodology

Markers for diversity analysis

The diversity analysis among the lentil genotypes was done using 43 RAPD primers and 73 SSR primers (Table. 9, 10). The RAPD primers were procured from the OPERON TECHNOLOGIES, USA. The SSRs used during this study were available for the chickpea genome and were validated in lentil genotypes since lentil's are quite close to chickpea for their evolutionary relationship.

General reaction conditions for RAPD

DNA isolation was done as per procedure described for pigeonpea in this Bulletin. PCR amplification was performed in 200 μl thin-wall tubes using a MJ Research PTC-200 thermocycler. Reaction solutions (10 μl) contained the 25 ng of genomic DNA template, 0.6 unit of *Taq* DNA polymerase (Bangalore Genei), 200 μM each of dATP, dCTP, dGTP and dTTP (Bangalore Genei) and 1.0 μM decamer primer. The PCR buffer consisted of 10 mM Tris-HCl/50 mM KCl/0.1 mg/ml gelatin, pH 8.3 (Bangalore Genei). Cycle conditions were , initial denaturation step at 94^{0}C for 3 min followed by 42 cycles of 94^{0}C for 1 min, 37^{0}C for 1 min and 72^{0}C for 2 min with a final extension at 72^{0}C for 7 min.

General reaction conditions for SSR

PCR amplification was performed in 200 μl thin-wall tubes using an MJ Research PTC-200 thermocycler. Reaction solutions (10 μl) contained the 25ng of genomic DNA template, 0.6 unit of *Taq*DNA polymerase (Bangalore Genei), 200 μM each of dATP, dCTP, dGTP and dTTP (Bangalore Genei), 1.0 μM each of forward and reverse primers. The PCR buffer consisted of 10 mM Tris-HCl/50 mM KCl/0.1 mg/ml gelatin, pH 8.3 (Bangalore Genei). Cycle conditions were, initial denaturation step at 94^{0}C for 3 min followed by 40 cycles of 94^{0}C for 1 min, 54^{0}C for 1 min. and 72^{0}C for 2 min with a final extension at 72^{0}C for 7 min.

Diversity analysis

Amplification products were resolved by electrophoresis on 1.2% agarose gel for RAPD and 2% agarose gel for SSR and visualized with UV illumination.

Statistical analysis

The banding patterns obtained with the DNA amplified with RAPDs and SSRs were analysed to estimate genetic relationships among the 31 Lentil genotypes. Amplified samples were scored for the presence (1) or absence (0) of homologous bands to create a binary matrix of the different RAPDs and SSRs genotyped by using Alpha DigiDoc software. A cluster analysis based on the similarity matrix was performed using the unweighted pair

group method with arithmetical averages (UPGMA). NTSYS–ps version 2.0 software was used for cluster analysis and then the dendrogram was prepared.

Table 9 : List of RAPD markers used for diversity analysis

Primer	Sequence	Maximum no. of bands	Polymorphic(P)/ Non polymorphic (NP)/ Not Amplified (NA)
OPAC –4	ACGGGACCTG	4	NP
OPAC-6	CCAGAACGGA	1	NP
OPAC-7	GTGGCCGATG	3	NP
OPAC-8	TTTGGGTGCC	7	P
OPAC -9	AGAGCGTACC	NA	NA
OPAC-10	AGCAGCGAGG	7	NP
OPAC-11	CCTGGGTCAG	6	P
OPAC-12	GGCGAGTGTG	4	P
OPAC-13	GACCCGATTG	NA	NA
OPAC-3	CACTGGCCCA	5	P
OPAC-18	TTGGGGGAGA	1	NP
OPAC-20	ACGGAAGTGG	1	NP
OPBC			
OPBC-1	CCTTCGGCTC	NA	NA
OPBC-2	ACAGTAGCGG	4	P
OPBC-3	GGCTTGACCT	4	P
OPBC-6	GAAGGCGAGA	4	P
OPBC-7	TGTGCCTGAC	5	P
OPBC-8	GGTCTTCCCT	3	P
OPBC-10	AACGTCGAGG	3	P
OPBC-11	TTTTGCCCCC	3	P
OPBC-12	CCTCCACCAG	5	P

OPBC-13	CCTGGCACAG	4	P
OPBC-14	GGTCCGACGA	4	NP
OPBC-15	CCAGACTCCA	5	P
OPBC-16	CTGGTGCTCA	5	P
OPBC-17	CCGTTAGTCC	6	NP
OPBC-18	GTGAAGGAGG	4	P
OPBC-19	ACAAGCGCGA	1	NP
OPBE			
OPBE-1	CACTCCTGGT	3	P
OPBE-2	ACGCCTGTAG	3	P
OPBE-3	TGGACTCGGT	6	P
OPBE-4	CCCAAGCGAA	7	NP
OPBE-5	GGAACGCTAC	9	P
OPBE-6	CAGCGGGTCA	7	P
OPBE-7	CCGTCCTATG	4	P
OPBE-8	GGGAAGCGTC	9	P
OPBE-9	CCCGCTTTCC	4	P
OPBE-10	AAGCGGCCCT	3	P
OPBE-11	GTCCTGCTGT	4	P
OPBE-14	CTTTGCGCAC	3	NP
OPBE-15	TTCGGCGATG	3	P
OPBE-16	CTCCACGACT	5	P
OPBE-17	GGGAAAAGCC	5	P
	Total	174	

Table10. List of the SSRs used for the diversity analysis of lentil

Primer	Annealing Temp.	% of gel	Band Size (bp)	Polymorphism (P)/ Non polymorphism(NP)
SSR-1	52-54ºc	2 % agarose	70, 95	P
SSR-2	52-54ºc	2% agarose	80, 100	P
SSR-4	54ºc	Metaphor	80-150	P
SSR-5	54ºc	2% agarose	30,75	P
SSR-6	54ºc	2% agarose	70,150	P
SSR-7	54ºc	2% agarose	70-90	P
SSR-9	54ºc	2% agarose	90	NP
SSR-10	54ºc	Metaphor	70,160	P
SSR-11	54ºc	2% agarose	30,50	P
SSR-12	54ºc	2% agarose	80,110	P
SSR-13	54ºc	2% agarose	75,80	P
SSR-14	54ºc	2% agarose	90,100	P
SSR-17	54ºc	Metaphor	60,75	NP
SSR-18	54ºc	2% agarose	50	NP
SSR-19	54ºc	2% agarose	60,145	P
SSR-21	54ºc	2% agarose	30, 50	P
SSR-22	54ºc	2% agarose	60,100	P
SSR-23	54ºc	Metaphor	40,85	P
SSR-24	54ºc	2% agarose	60	NP
SSR-27	54ºc	2% agarose	55	NP
SSR-28	54ºc	Metaphor	80,110	P
SSR-29	54ºc	2% agarose	60,170	P
SSR-30	54ºc	2% agarose	60,125	P
SSR-32	54ºc	2% agarose	40,60	P

SSR-34	50ºc	2% agarose	80,100	P
SSR-35	50ºc	2% agarose	70,180	P
SSR-36	50ºc	2% agarose	90,150	P
SSR-39	54ºc	2% agarose	180,200	P
SSR-40	54ºc	2% agarose	50,75	P
SSR-41	54ºc	2% agarose	60,100	P
SSR-42	54ºc	2% agarose	30,60	P
SSR-49	54ºc	2% agarose	40,70	P
SSR-53	54ºc	2% agarose	60,75	P
SSR-54	54ºc	Metaphor	60,80	P
SSR-55	54ºc	2% agarose	70,80	P
SSR-56	54ºc	2% agarose	200,250	P
SSR-57	54ºc	2% agarose	330,430	P
SSR-58	54ºc	2% agarose	75	NP
SSR-60	54ºc	2% agarose	75,100	P
SSR-61	54ºc	2% agarose	70,120	P
SSR-65	54ºc	2% agarose	30,65	P
SSR-71	54ºc	2% agarose	55,165	P
SSR-75	54ºc	2% agarose	30,60	P

Significant Findings

- Among 43 RAPD primers tested, 40 RAPD primers amplified resulting in at least one band, whereas 3 markers did not amplify. Among the primers that were amplified, 72% (29/40) resulted in polymorphic bands (Fig 10) where as 27.5% (11/40) gave monomorphic bands (Fig 9). A total of 174 bands were observed using RAPD primers (Table. 9).
- All the 43 SSRs (SSRs and STMSs) tried during the experiment, 13.9% (6/43) were non-polymorphic where as all the other SSRs (37/43) gave polymorphisms (Fig 10 and 11). The experiments were reproducible.

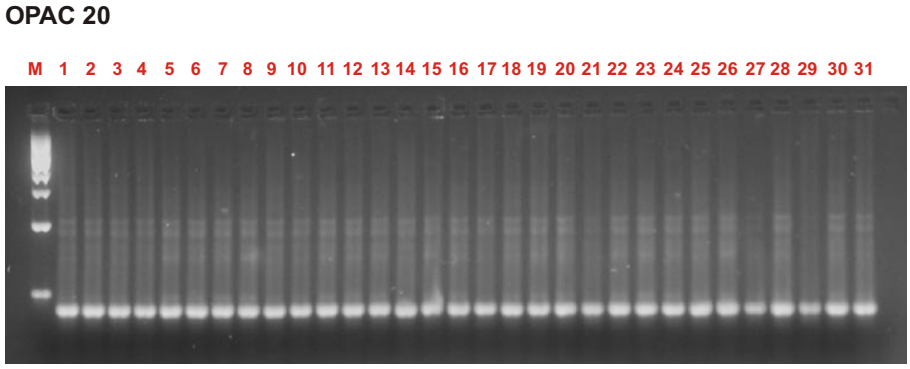


Fig. 9: Non-Polymorphic banding pattern of 31 lentil genotypes (1-31) using RAPD primer OPAC-20, lane 1: 500bp ladder

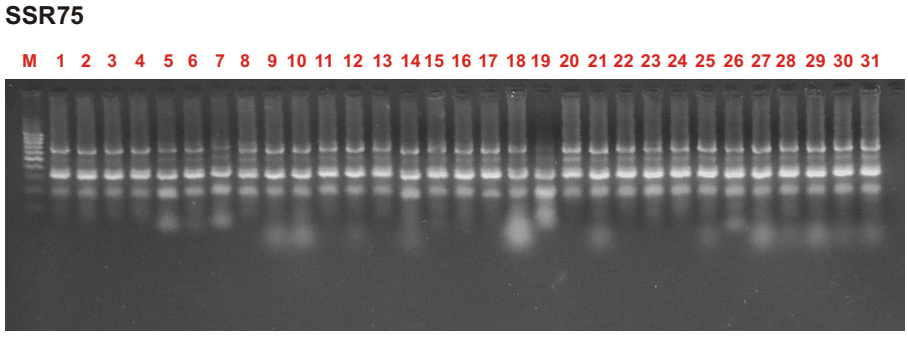


Fig. 10: Polymorphic banding pattern of 31 lentil genotypes (1-31) using SSR primer SSR-75, lane 1: 100bp ladder

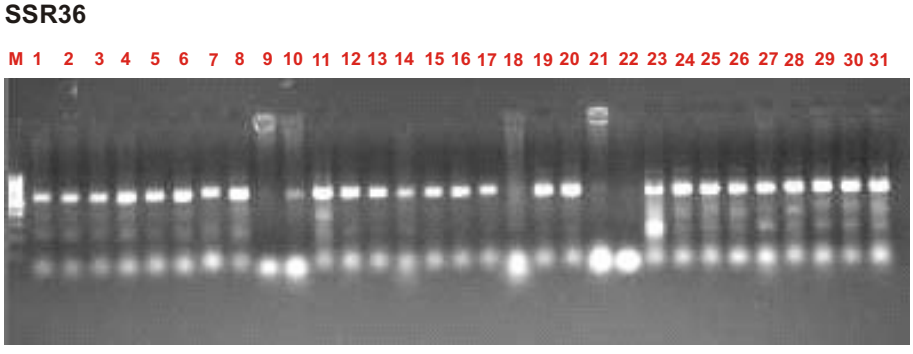


Fig. 11: Polymorphic banding pattern of 31 lentil genotypes (1-31) using SSR primer SSR-36, lane 1: 100bp ladder

- Cluster analysis was performed using SSR and RAPD data separately. The dendrogram derived from RAPD data alone clustered together some lines with similar genetic background. The dendrogram based on SSR data confirms the association between the above mentioned genotypes.
- The genotypes viz., LH 84-8 and PL-639 have one parent i.e., L-9-12 in common. In RAPD analysis, these three genotypes are quite far off from one another, where as in the SSR analysis, the genotypes L-9-12 and PL 639 are quite close. This indicates that the SSRs are markers of choice for the diversity analysis.
- The fingerprint pattern of the 31 genotypes with selected SSR markers are given in Table 10. This can be helpful in identifying a lentil cultivar, supported by morphological and related characters.
- Neither from the RAPD nor from the SSR data, it is conclusive that Ranjan is a mutant of B77 since there is a high degree of dissimilarity between them.
- The estimates of genetic similarity based on Jaccards' coefficients ranged from 0.49 to 0.87 using RAPDs where as for SSRs it is 0.65 to 0.94. Hence both RAPD and SSR markers are quite useful for the diversity analysis among the Indian lentil cultivars.

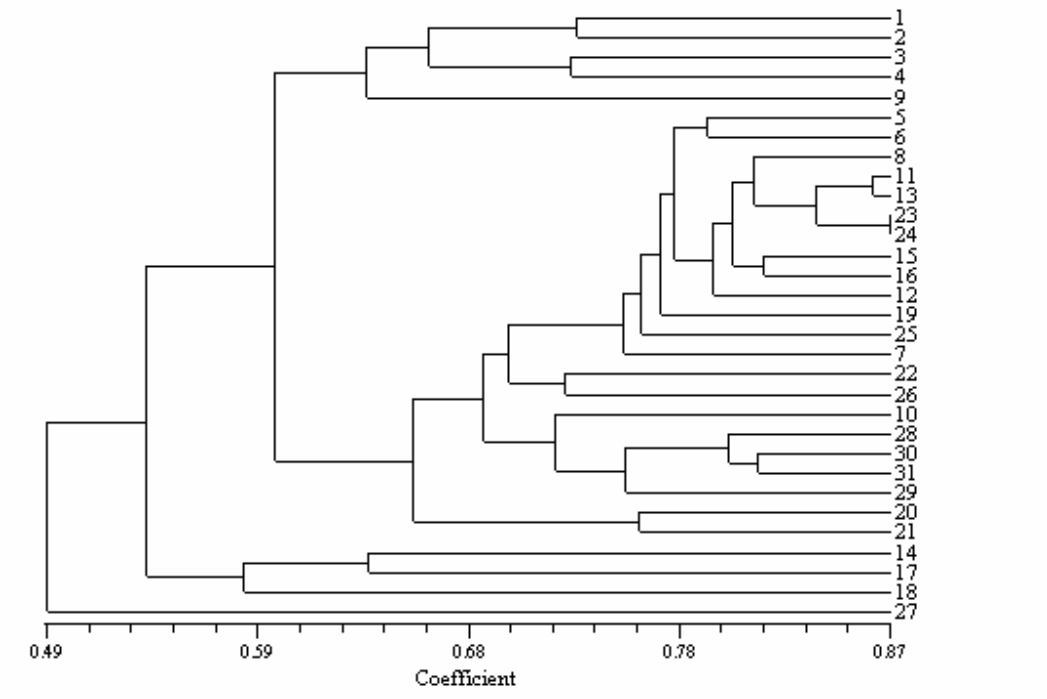


Fig. 12. Dendrogram developed by use of RAPD markers

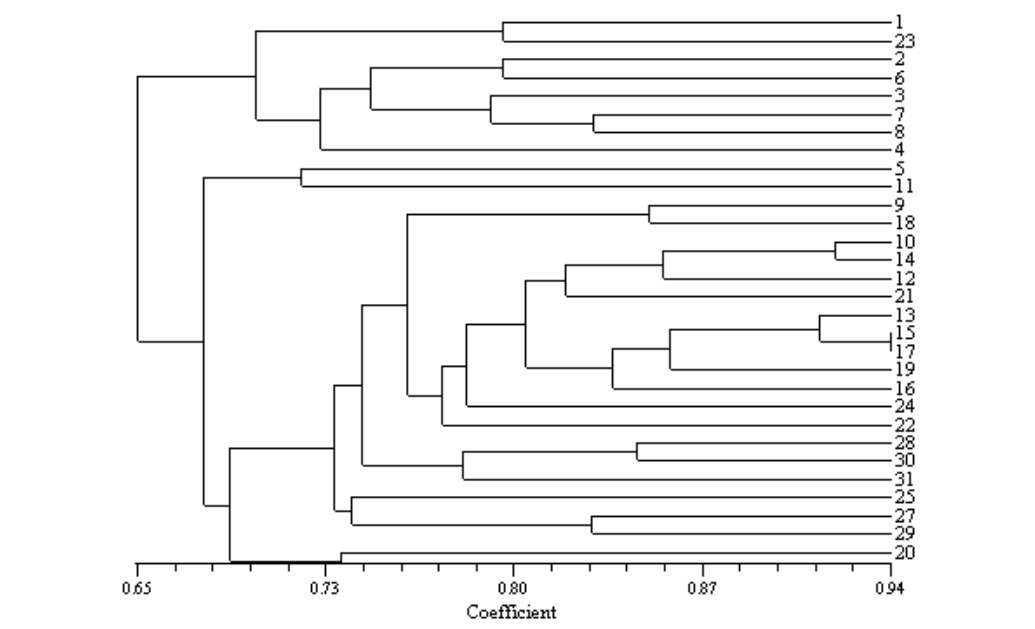


Fig. 13. Dedrogram produced using SSR markers

- Many exotic lines like Masor and Precoz do not form separate clusters (Fig 12 & 13). It indicates the existence of diversity among the lentil varieties to be quite low, and introgression of genome(s) into different backgrounds during development of varieties took place.

FIELDPEA

Background

Pea (*Pisum sativum* L.) is one of the most important food legumes in the world not only for its very old history of domestication but also for its versatile use as vegetable, pulse and feed. Its area and production ranks second amongst the cool season pulses in the world and the third largest area of pea exist in India after Canada and Russia (Khan & Dixit, 2001). Peas are grown all over India and Indian sub-continent and consumed as green vegetable, *chat* (spicy dish), *chhola* (whole grain), *dhal* (pulses) and flour, thus contributes significantly to market economy. It occupies an area of 0.77 m ha and produces 0.71 m tones of grain with productivity of 915 kg/ha(2005-06).

Determination of genetic diversity in a gene pool is the key to crop improvement. For estimation of genetic diversity and establishment of varietal identity, various types of marker systems are being used by plant breeders (*viz.* morphological, biochemical or molecular). Molecular markers are numerous, reliable, independent of environmental interactions, do not show stage specific expression of characters and provide high degree of polymorphism and thus preferred over other marker systems. Several molecular techniques can be used for varietal identification and diversity studies which includes restriction fragment length polymorphism (RFLP, Sambrook *et al*., 1989), random amplified polymorphic DNA (RAPD, Williams *et al*., 1990), simple sequence repeats (SSRs, Jacob *et al.,* 1991), amplified fragment length polymorphism (AFLP, Vos *et al*., 1995) and more recently SSAPs (sequence specific amplification polymorphism, Waugh et al., 1997), IRAPs (inter retrotransposon amplified polymorphism) or REMAPs (retro transposon microsatellite amplified polymorphism, Kalendar *et al.,* 1999). Among these techniques, RAPD is not only a multi locus marker (Karp *et al*., 1997) but also the fastest and simplest. Due to these advantages over other marker systems, RAPD has been applied successfully for genetic diversity analysis in many important crop species including pea.

Therefore, in order to obtain an overall picture of the genetic diversity among the important released pea varieties in India, which covers a great extent of gene pools, RAPD marker assays have been employed. Moreover, the efficiency of the RAPD marker systems to identify the varieties were also judged.

Material

A total of 24 elite released varieties of pea cultivated across India and representing different morphological variations, agro climatic zones and parentage were selected for molecular polymorphism studies (Table 11). All the cultivars were collected from core collection maintained by the concerned breeder at Indian Institute of Pulses Research, Kanpur, India.

Table 11: List of fieldpea varieties and their pedigree used for molecular diversity analysis

Sl. No.	Name	Parentage	Characteristics
1.	KFP 103	KPMR 83 X KPMR 9	Tall type, light green leaves
2.	PG 3	T 163 X Bonnevilla	Dwarf type, blue-green leaves
3.	Rachna	T 163 X T 10	Tall type, green leaves
4.	IPF 99-25	PDPD 8 X Pant P 5	Tall type, green leaves
5.	Pant P 5	T 10 X T 163	Tall type
6.	JM 6	Local yellow Botri X (6588-1 X 46 C)	Tall type
7.	Jayanti	HFP 4 X PG 3	Dwarf type
8.	KPMR 522	KPMR 156 X HFP 4	Dwarf type, leaflet less
9.	KPMR 144-1	Rachna X HFP 4	Dwarf type
10.	DMR 7	6587 X L 116	Tall type
11.	Ambika	DMR 22 X HUP 7	Tall type
12.	VL 1	Selection from Miller	Tall type
13.	VL 3	Old Sugar X Wrinkled Dwarf	Dwarf type, blue-green leaves, dual purpose
14.	B 22	Selection of local material from Berhampore (State of West Bengal, India)	Tall type, blue flower, blue-green leaves
15.	JP 885	(T 163 X 6588-1) X 46C	Tall type and erect
16.	Swati	Flavanda X HFP 4	Dwarf type, leaflet less
17.	Subrita	Rachna X JP 885	Tall type
18.	HUP 2	(Alfaknud X C 5064) X S 143	Tall type, leaflet less
19.	DDR 44	HFP 4 X KPMR 157	Dwarf type, and short duration
20.	HFP 8909 (Uttara)	EC 109185 X HFP 4	Dwarf type, leaflet less
21.	KPMR 400	Rachna X HFP 4	Dwarf type, leaflet less
22.	HFP 4	T 163 X EC 109196	Dwarf type, leaflet less
23.	HUDP 15	(PG 3 X S143) X FC 1	Dwarf type, leaflet less
24.	IPFD 99-13	HFP 4 x LFP 80	Dwarf type, leaflet less

Methodology

DNA extraction

Genomic DNA from each pea variety was isolated from bulked leaf samples (approximately 1 g each) plucked from young seedlings of one month age. Isolation of DNA was done based on the modified protocol of Guillemant and Laurence (1992). Pooled leaf samples were ground to a very fine paste using the grinding buffer [100 mM sodium acetate, pH 4.8; 500 mM Nacl; 50 mM EDTA, pH 8.0; 50 mM Tris, pH 8.0; 2% PVP (MW 10000); 1.4% SDS] and incubated at 65°C for 30 minutes followed by 65°C for 15 minutes after addition of 0.6 volume of 10 M ammonium acetate. Centrifugation was done for 10 minutes at 10 K RPM. Then 0.6 volume of chilled iso-propanol was mixed with the supernatant and kept at -20°C for 60 minutes for DNA precipitation. DNA was pelleted out, washed twice with 70% ethanol and dissolved in TE (10 mM Tris, 1.0 mM EDTA, pH 8.0). Dissolved DNA solution was extracted with phenol: chloroform: iso-amyl alcohol (25:24:1) and RNA was removed by RNAse treatment @ 4 µl/ml of grinding buffer from stock of 50mg/ml of RNAse) at 37 °C for 1 hr. Dissolved DNA (in TE buffer) was further extracted twice with chloroform: iso-amyl alcohol (24:1). DNA was re-precipitated and dissolved in TE buffer. Purified DNA was checked for its quality and quantity by 0.8% agarose gel electrophoresis using uncut lambda (λ) DNA as standard (300 ng/µl). Dilution of the DNA solution was done using TE buffer to a concentration of approximately 25 ng/2 µl for use in PCR analysis.

DNA amplification by PCR

PCR amplification was carried out in 0.2 ml thin-wall PCR tubes using a What man Biometra (model T1 Thermocycler) thermal cycler. A total of 80 RAPD primers were screened in the present study and unambiguous DNA profile was generated by 60 primers. Polymerase chain reaction (PCR) mixture of 25 µl contained 25 ng of genomic DNA template, 0.6 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 25 ng of decamer primer (Operon Technologies, Alameda, CA, USA), 2.5 µl of 10 X PCR assay buffer (50 mM KCl, 10 mM Tris – Cl, 1.5 mM $MgCl_2$) and 0.25 µl of pooled dNTPs (100 mM each of dATP, dCTP, dGTP and dTTP from Fermentas Life Sciences, USA). PCR cycle conditions were, initial denaturing step at 94 °C for 3 min followed by 44 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min. In the last cycle, primer extension at 72°C for 7 min was provided.

Documentation of agarose gels

PCR products were electrophoretically separated on a 1.5% agarose gel containing ethidium bromide using 1X TBE buffer (pH 8.0). The amplified products were visualized and photographed under UV light source. O'Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas Life Sciences, USA) was used as molecular weight marker.

Data analysis:

DNA bands were scored '1' for its presence and '0' for its absence for each primer-genotype combination. These binary data matrix was then utilized to generate genetic similarity data among genotypes. Only unambiguous bands were scored for the estimation of genetic similarity according to Jaccard's coefficient of similarity. Based on these data, UPGMA (unweighted pair group method using arithmetic averages) clustering was carried out using the software package NTSYS-pc (Rohlf, 1992). The expected heterozygosity for a genetic marker (H_n) was calculated by $H_n = 1\text{-}pi^2$ (pi is the allele frequency of the ith allele (Nei, 1987). By using the values of H_n, H_{av} (the arithmetic mean heterozygosity) was calculated by $H_{av} = H_n/n$ (n = number of markers or loci analysed, Powell *et al.* 1996). The average heterozygosity for polymorphic markers $(H_{av})p$ was derived as $(H_{av})p = H_n/np$ (np = no. of polymorphic markers or loci). Marker index (MI) was also calculated as $MI = E (H_{av})p$ (E is effective multiplex ratio and measured by nb where b is the fraction of polymorphic marker or loci). To estimate the probability of DNA fingerprinting of any two cultivars being identical by chance as described by Ramakishana *et al.* (1994), average similarity index for all pair wise comparison (X_D) was done by employing the formula $(X_D)^n$, where X_D= average similarity index and n = average number of amplified products per cultivar.

Salient Findings

RAPD Polymorphism

- Out of total 80 primers tried in the PCR amplification, 60 primers showed clear and unambiguous amplification while rest of the primers did not give amplification in several reactions tried or produced very faint or fuzzy lanes (Table 12). Scorable 60 RAPD primers led to amplification of 579 fragments ranging from about 4000 bp (by OPP 17) to 180 bp (OPBA 06), out of which 433 (74.8%) bands were found to be polymorphic.

Table 12: Analysis of the amplified DNA fragments obtained with RAPD primers in various cultivars of fieldpea

Primer	Total no. of bands amplified (x)	No. of polymorphic bands (y)	Percent polymorphism (x/y) x 100	No(s). and mol. wt. of unique profile in base pairs (bp) with sl. no. of variety
OPP 01	13	10	76.9	1 (875 bp,24)
OPP 02	07	04	57.1	-
OPP 03	06	04	66.7	1 (500 bp,18)
OPP 04	13	13	100	3 (3000 bp,24; 2000 bp,18; 250 bp,17)

OPP 05	07	05	71.4	1 (1275 bp,22)
OPP 06	09	06	66.7	1 (950 bp,14)
OPP 07	12	07	58.3	1 (1275 bp,12)
OPP 08	10	08	80	1 (650 bp,15)
OPP 09	07	06	85.7	2 (1700 bp,13; 700 bp,7)
OPP 10	15	12	80	2 (1750 bp,21; 1350 bp,15)
OPP 11	10	09	90	-
OPP 12	09	07	77.7	1 (600 bp,12)
OPP 13	22	21	95.5	1 (1200 bp,2)
OPP 14	16	13	81.3	-
OPP 15	14	10	71.4	2 (1650 bp,22; 1400 bp,6)
OPP 16	12	10	83.3	1 (510 bp,16)
OPP 17	14	11	78.6	-
OPP 19	12	09	75	2 (1825 bp,14; 575 bp,14)
OPBA 01	02	01	50	-
OPBA 02	06	05	83.33	2 (2600 bp,22; 350 bp,22)
OPBA 03	09	07	77.78	-
OPBA 04	13	12	92.3	-
OPBA 05	07	06	85.71	-
OPBA 06	12	04	33.3	-
OPBA 07	11	05	45.5	-
OPBA 08	12	09	75	2 (600 bp,12; 360 bp,2)
OPBA 09	15	13	86.7	1 (1950 bp,22)
OPBA 10	08	07	87.5	-
OPBA 11	14	12	85.7	-
OPBA 12	08	04	50	-
OPBA 14	08	05	62.5	1 (475 bp,7)
OPBA 15	07	05	71.4	-

OPBA 16	07	05	71.4	1 (275 bp,22)
OPBA 18	10	07	70	-
OPBA 19	09	07	77.8	-
OPBA 20	08	06	75	1 (800 bp,23)
OPAZ 01	07	06	85.7	1 (700 bp,3)
OPAZ 03	04	03	75	2 (2900 bp,23; 800 bp,13)
OPAZ 04	10	05	50	-
OPAZ 14	08	04	50	-
OPAZ 16	09	06	66.7	-
OPAZ 19	08	07	87.5	-
OPAQ 05	13	07	53.9	1 (725bp,12)
OPAQ 06	08	06	75	-
OPAQ 09	06	05	83.3	-
OPAQ 13	14	08	57.1	-
OPAQ 15	14	12	85.7	1 (650 bp,18)
OPAQ 16	05	04	80	2 (2900 bp,21; 2500 bp,14)
OPAQ 18	09	04	44.4	-
OPAQ 19	08	04	50	-
OPAQ 20	09	01	11.1	-
OPH 01	02	02	100	-
OPH 02	15	15	100	2 (1250 bp,12; 340 bp,12)
OPH 03	12	10	83.3	1 (450 bp,18)
OPH 04	04	02	50	-
OPH 05	06	03	50	1 (400 bp,12)
OPH 07	07	07	100	2 (1900 bp,7; 1450 bp,14)
OPH 08	11	11	100	1 (685 bp,14)
OPH 09	08	08	100	1 (475 bp,19)
OPH 20	08	08	100	-
Total	579	433		43

(N.B. The RAPD primers OPP 18,20; OPBA 13,17; OPAZ 02,05, 13,15,17,18, 20; OPAQ 07, 08, 10,11,12,14,17 and OPH 06, 10 produced fuzzy lanes or did not amplify; digits given in the parenthesis depicts molecular weight of the unique band(s) in base pairs and serial no. of the variety corresponding to Table 11, respectively).

- The level of polymorphism ranged from 11.1% (by OPAQ 20, where 8/9 bands found to be monomorphic) to 100% (by OPP 04 and OPH 01, 02, 07, 08, 09 and 20, where the number of amplified DNA fragments were found to be 13, 02, 15, 07, 11, 08 and 08, respectively).
- Maximum number of 22 amplified products was obtained by primer OPP 13. Moreover, OPP 14 produced 16 bands followed by OPP 10, OPBA 09 and OPH 02 with 15 bands each. The primers OPBA 01 and OPH 01 both amplified a minimum of 2 bands each.
- On an average 9.65 bands per primer were obtained and 26 primers out of 60 primers (43.3%) used in the study produced DNA bands more than the average value of 9.65. Forty three RAPD products produced by 32 RAPD primers were recorded as variety specific. DNA amplification pattern as detected by some of the RAPD primers in the elite pea cultivars has been provided in Figure 14.

Heterozygosity and marker index:

- Heterozygosity was calculated for the 579 amplified products obtained by employing 60 RAPD primers across the varieties. The H_{av} and $(H_{av})p$ were found to be 0.496 and 0.663, respectively, whereas the marker index (MI) value was obtained to be 4.787 (Table 13).

Table 13: Efficiency of RAPD primer system in detecting genetic diversity in fieldpea cultivars

No. of RAPD primers analyzed	Av. % of polymorphism detected	Av. no. of bands/ primer	H_{av}	$(H_{av})p$	MI
60	74.8	9.65	0.496	0.663	4.787

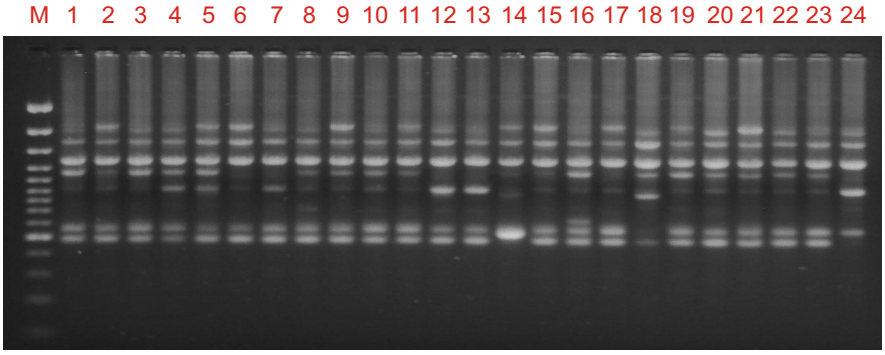
Genetic relationship and genotyping:

- The degree of genetic relatedness among cultivars evolved from various geographical areas, morphological parameters and parentages varied considerably (0.599 to 0.873) (Table 14). Highest similarity (0.873) was measured between tall type DMR 7 and Ambika. Very high degree of similarity was also found between Rachna and IPF 99-25 (0.848) and between KPMR 144-1 and DRR 44 (0.844). It was also evident that more than 80% similarity have been obtained between the pairs of Rachna and DMR 7; IPF 99-25 and DMR 7; Pant P 5 and JP 885; KPMR 522 and KPMR 144-1; KPMR 522 and DRR 44; HFP 8909 and HFP 4. On the other hand, least similarity (0.599) was

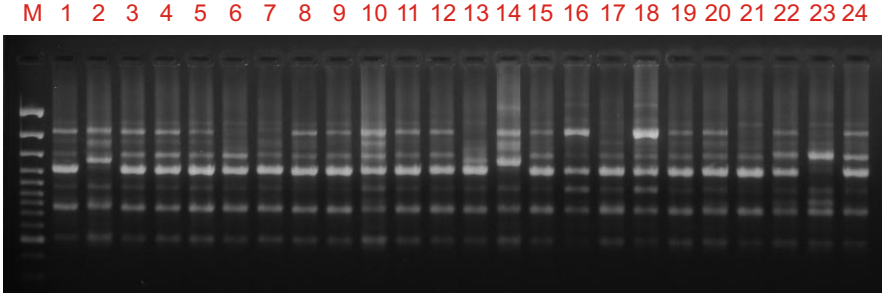
Table 14: Genetic similarity matrix of 24 elite cultivars of pea

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	1.00																							
2	0.708	1.00																						
3	0.775	0.712	1.00																					
4	0.773	0.698	0.848	1.00																				
5	0.759	0.689	0.772	0.775	1.00																			
6	0.702	0.690	0.735	0.734	0.753	1.00																		
7	0.661	0.663	0.688	0.671	0.680	0.671	1.00																	
8	0.707	0.663	0.758	0.762	0.734	0.735	0.680	1.00																
9	0.720	0.672	0.762	0.760	0.751	0.729	0.654	0.836	1.00															
10	0.762	0.723	0.801	0.803	0.779	0.756	0.677	0.755	0.788	1.00														
11	0.742	0.746	0.794	0.782	0.768	0.770	0.682	0.755	0.772	0.873	1.00													
12	0.648	0.642	0.706	0.697	0.729	0.698	0.675	0.675	0.693	0.695	0.722	1.00												
13	0.629	0.688	0.682	0.669	0.682	0.724	0.674	0.691	0.704	0.680	0.716	0.714	1.00											
14	0.638	0.615	0.651	0.648	0.693	0.657	0.592	0.648	0.679	0.686	0.686	0.670	0.651	1.00										
15	0.713	0.687	0.754	0.762	0.812	0.789	0.677	0.721	0.753	0.790	0.784	0.735	0.725	0.681	1.00									
16	0.688	0.641	0.701	0.701	0.715	0.688	0.693	0.753	0.761	0.722	0.717	0.688	0.700	0.621	0.721	1.00								
17	0.692	0.702	0.725	0.719	0.729	0.730	0.666	0.688	0.701	0.765	0.799	0.670	0.691	0.665	0.759	0.674	1.00							
18	0.634	0.617	0.678	0.670	0.683	0.657	0.661	0.697	0.687	0.680	0.690	0.636	0.651	0.626	0.668	0.700	0.701	1.00						
19	0.712	0.677	0.753	0.775	0.757	0.739	0.698	0.811	0.844	0.769	0.773	0.702	0.714	0.671	0.763	0.776	0.729	0.715	1.00					
20	0.680	0.726	0.734	0.698	0.694	0.717	0.740	0.739	0.720	0.727	0.750	0.702	0.724	0.649	0.726	0.753	0.711	0.693	0.776	1.00				
21	0.730	0.624	0.715	0.724	0.724	0.707	0.610	0.778	0.827	0.722	0.717	0.657	0.668	0.665	0.730	0.696	0.696	0.656	0.787	0.684	1.00			
22	0.649	0.673	0.697	0.676	0.680	0.663	0.698	0.698	0.680	0.687	0.700	0.651	0.688	0.599	0.678	0.724	0.684	0.679	0.729	0.820	0.637	1.00		
23	0.658	0.647	0.676	0.672	0.672	0.641	0.686	0.704	0.722	0.696	0.683	0.654	0.670	0.622	0.661	0.722	0.667	0.658	0.718	0.714	0.671	0.718	1.00	
24	0.709	0.635	0.699	0.695	0.673	0.682	0.664	0.722	0.731	0.675	0.697	0.656	0.649	0.633	0.692	0.726	0.668	0.660	0.741	0.727	0.727	0.700	0.674	1.00

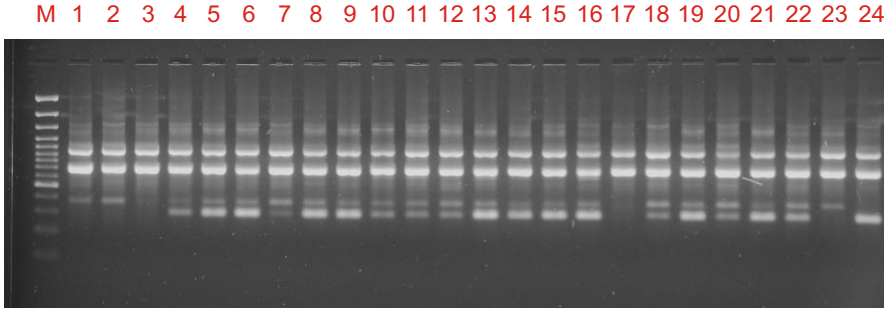
Key to individuals: 1. KPF 103, 2. PG 3, 3. Rachna, 4. IPF 99-25, 5. Pant P 5, 6. JM 6, 7. Jayanti, 8. KPMR 522, 9. KPMR 144-1, 10. DMR 7, 11. Ambika, 12. VL 1, 13. VL 3, 14. B 22, 15. JP 885, 16. Swati, 17. Subrita, 18. HUP 2, 19. DRR 44, 20. HFP 8909, 21. KPMR 144-1 22. HFP 4, 23. HUDP 15, 24. IPFD 99-13.



(a)



(b)



(c)

Figure14: RAPD profile of pea varieties obtained with primers OPP 08 (a), OPP 16 (b) and OPBA 19 (c). Serial number of the varieties corresponds to table 11. M=Standard DNA marker, 100 bp DNA ladder plus.

found between tall type land race B 22 and dwarf type HFP 4. B 22 also showed considerable amount of diversity with PG 3, Rachna, IPF 99-25, Swati, HUP 2, HFP 8909, HUDP 15 and IPFD 99-13 (average similarity between 62-65%).

- Multivariate (cluster) analysis of the genetic similarity data grouped the cultivars into two major clusters with two sub-groups in each cluster (1A, 1B and 2A, 2B, respectively, (Fig 15). The sub-group 1A is consisted of all the tall type cultivars and the sub- group 1B includes all the dwarf type cultivars. The sub group 2A of the major cluster 2 contains four dwarf type cultivars while 2B have two varieties (*viz.* VL 1 and VL 3). Three cultivars PG 3, HUP 2 and B 22 positioned themselves separately from any sub-cluster and all of them are either morphologically variable from others with different geographical distribution or having exotic origin.

Identification of genotypes:

- The probability of identical match by chance was found to be 2.96×10^{-53} (Table 15) which indicates that by employing 60 RAPD primers, approximately 3×10^{53} pea genotypes can be precisely identified.

Table 15. Analysis of DNA fingerprints using 60 RAPD primers

Av. bands for each variety ± SD	Av. Similarity index (X_D) ± SD	Probability of identical match by chance $(X_D)^n$
356.08 ± 8.57	0.712 ± 0.075	2.96×10^{-53}

- The data set using RAPD as a marker system enabled to identify at least 10^{53} genotypes unambiguously (Table 15). Moreover, for further studies, 42 unique products across cultivars as detected by the RAPD analysis (Table 12) could be converted into CAPS or SCAR marker for varietal confirmatory tests.

Analysis of polymorphism:

- In present study, 74.8% fragments were found to be polymorphic as compared to 55.7% obtained by Simioniuc *et al.* (2002) in 21 released German cultivars by employing 20 RAPD primers. Moreover, estimated genetic similarity obtained by the same workers varied from 0.80 to 0.94 (mean 0.88), whereas in our studies it varied from 0.599 to 0.873 (mean 0.712) because of the wide range of geographical distribution and pedigree of the selected genotypes.
- The H_{av} value was found to be higher (0.496) and thus proving the efficiency of RAPD as a marker system in detecting heterozygosity in pea. Based on allozyme diversity, the

estimated mean heterozygosity (H_{av}) have been reported to be very low in self pollinating leguminous crop cowpea (0.027; Pasquet, 2002) and moderately higher in wild lentil (0.342; Huh & Huh, 2001). When RAPD was used as marker system, the H_{av} value was found to be 0.203 in *Trigonella foenum–graecum* and 0.346 in *Trigonella caerula* (Dangi *et al*. 2004).

Phylogenic relationship:

- A total of 60 RAPD primers developed altogether 579 bands and among them 433 was found to be polymorphic (74.8%). This result is comparable with the data obtained by Simioniuc *et al*., (2002) where 55.7% polymorphic bands were obtained by using 20 RAPD primers in 21 German pea cultivars.
- The average similarity index of 0.71 between cultivars suggests that the genetic diversity among the pea cultivars is moderate. The average number of bands developed by the 60 RAPD primers (9.65) and the average percent polymorphism (74.8) indicated the efficiency of RAPD primers in detecting polymorphism among cultivars.
- Least similarity was obtained between the tall land race B 22 (a selection from eastern part of India) and dwarf, leafletless HFP 4 which is a cross between T 163 and an exotic collection. B 22 also showed significant variation from dwarf type PG 3 and tall type Rachna where T 163 was found to be a common parent. Close relationship was obtained between tall types DMR 7 and Ambika (DMR 22 is one of the parents). Moreover, KPMR 144-1 and DDR 44 also showed very high degree of similarity where HFP 4 is a common parent in both the cultivars.
- From the cluster analysis it was clearly evident that the major cluster is constituted by 2 subclusters (1A and 1B). The biggest sub-cluster (1A) includes all the tall type cultivars barring HUP 2 (generated from exotic cultivar Alfakund) and B 22 (land race). They all have T163 as a parent (directly or indirectly) in their pedigree. Both HUP 2 and B 22 positioned themselves away from any core cluster because of wide geographical distribution of their parents. The cluster 1B is constituted by 5 dwarf type cultivars having parental background of HFP 4 which has been evolved from T 163 and EC 109196.
- It was evident from the subcluster 2A that all the four dwarf genotypes have either HFP 4 genotype or PG 3 as common parent. Again, if the pedigree analysis of HFP 4 and PG 3 is done, the parent T 163 is the common one. Two cultivars VL 1 and VL 3 formed fourth cluster (2 B) with exotic background and grown in temperate hilly northern region of India. Among the ancestors, T 163 was the most frequently used parent followed by EC 109196 and T 10. T 163 was mostly used for its wider adaptability whereas T 10 was used as donor parent for powdery mildew resistance (Fig. 15). EC 109196 was used as a source for afila and dwarf plant type. Most of the other ancestors

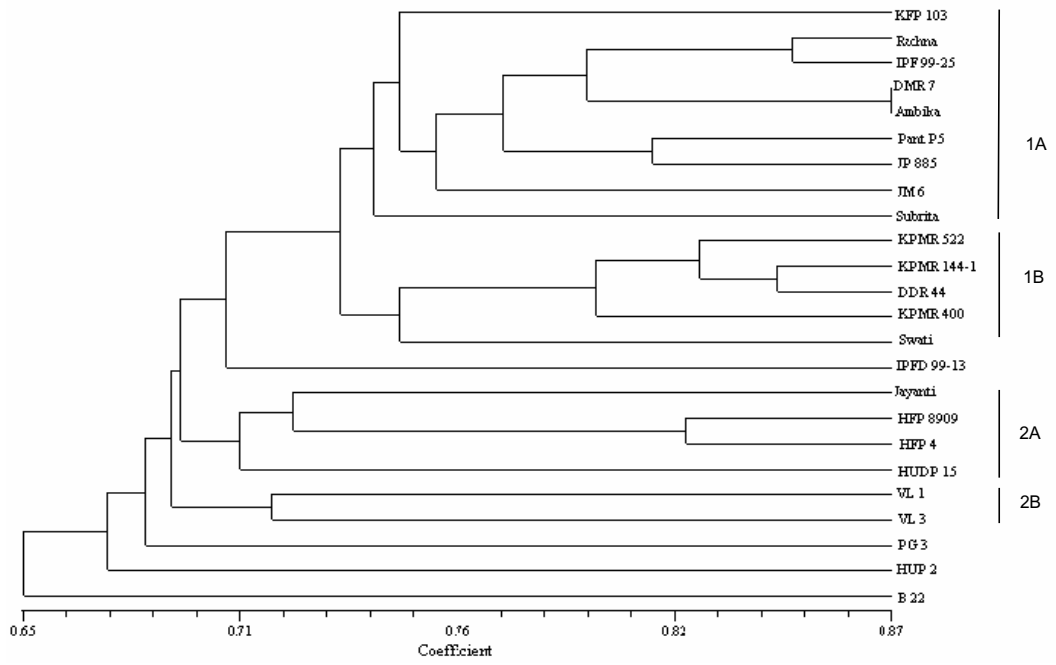


Figure 15: Dendrogram of fieldpea varieties, constructed using UPGMA based on 60 RAPD primers. The major clusters and sub-clusters are indicated on right margin.

were used as donor parent of single gene for disease resistance, particularly powdery mildew or plant type (afila or dwarf plant).

- All the primers showed polymorphism with a range between 11.1% to 100%. Out of 579 amplified products, 433 showed polymorphism (74.8%). On an average, 9.65 bands were amplified per primer.
- Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped all the tall type varieties together, whereas, dwarf types formed two different clusters based upon their pedigree.
- The arithmetic mean heterozygosity (H_{av}) value and marker index (MI) was found to be 0.496 and 4.787, respectively, thus indicated the efficiency of RAPD as a marker system. Moreover, probability of identical profiles by chance suggested that about 10^{53} genotypes can be unambiguously distinguished by employing 60 RAPD primers.

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DNA profile of individual cultivars of chickpea for selected RAPDs

Genotype	AZ- 1 A: 980, B: 1210	AZ- 4 A: 1200 B: 1542	AZ- 9 A: 488 B: 1010 C: 1240	AZ- 10 A: 500 B: 700 C: 1000	AZ-12 A: 525 B: 840	AM- 3 A: 500 B: 600 C: 1650	AM- 11 A: 350 B: 600 C: 780	AM- 13 A: 400 B: 700	AM- 14 A: 250 B: 600	AM-16 A: 500 B: 550	AM-16 A: 700 B: 900	AM-16 A: 1100 B: 1200 C: 1280	AC-9 A: 200 B: 370 C: 600
1	B	A	AB	B	B	ABC	ABC	A	A	A	B	AB	A
2	AB	B	AB	A	AB	ABC	AC	A	A	A	B	AB	AB
3	AB	B	AB	C	AB	ABC	AC	A	A	A	AB	AB	C
4	B	B	AB	BC	B		AC	A	A	A	AB	B	-
5	B	A	AB	ABC	AB	ABC	ABC	AB	A	AB	B	C	C
6	B	A	AB	ABC	AB	ABC	AC	AB	A	AB	AB	BC	AC
7	B	B	AB	ABC	B	ABC	AC	A	AB	AB	B	ABC	A
8	AB	B	AB	ABC	B	BC	AC	A	A	A	B	AC	A
9	B	B	AB	AB	B	BC	AC	A	A	A	B	AC	A
10	B	B	AB	AB	B		ABC	A	A	A	B	AC	A
11	B	B	AB	C	B	C	AC	A	A	A	B	AC	AB
12	AB	B	ABC	BC	B	BC	AC	A	A	A	B	AC	A
13	AB	A	ABC	AC	AB	ABC	AC	A	A	A	B	AC	AB
14	AB	B	ABC	ABC	B	BC	AC	A	A	A	B	AC	A
15	AB	B	ABC	ABC	AB	ABC	AC	A	A	A	B	AC	AB
16	AB	B	ABC	B	AB	ABC	ABC	AB	A	A	B	B	AB
17	AB	B	ABC	ABC	B	-	ABC	A	A	A	B	AB	C
18	AB	B	AB	ABC	AB	ABC	AC	A	A	AB	B	AB	C
19	B	B	AB	ABC	AB	ABC	C	A	A	AB	AB	A	AB
20	B	B	AB	AB	AB	ABC	AC	A	A	A	AB	A	ABC
21	B	B	ABC	B	AB	ABC	BC	A	A	AB	AB	B	ABC
22	AB	A	ABC	AB	AB	ABC	ABC	A	A	A	B	C	AB
23	AB	A	ABC	AB	AB	ABC	AB	A	A	AB	B	AB	AB
24	AB	B	AB	AC	AB	ABC	AC	A	AB	AB	B	AB	AB
25	A	B	AB	AC	AB	ABC	AC	A	A	AB	B	AB	A
26	B	B	ABC	AB	AB	AC	AC	A	A	AB	AB	AB	AB
27	B	B	ABC	AB	B	BC	AC	A	A	AB	B	AB	AB
28	B	B	ABC	AB	B	-	AC	A	A	A	B	AB	AB
29	B	B	AB	C	B	C	AC	A	A	A	B	A	ABC
30	B	B	ABC	B	AB	AC	A	A	A	A	B	ABC	AB

31	B	B	AB	BC	A	ABC	AC	A	A	AB	B	ABC	AB
32	B	B	AB	AC	AB	ABC	AC	A	A	AB	B	AC	AB
33	AB	B	AB	C	AB	AC	AC	A	A	AB	B	AC	A
34	B	B	AB	A	AB	A	AC	A	A	A	B	ABC	A
35	B	B	ABC	AC	AB	AB	A	A	A	AB	AB	ABC	ABC
36	B	AB	AB	AC	B	B	AC	A	AB	A	AB	AB	AB
37	B	AB	AB	AC	B	BC	AC	A	AB	A	AB	AB	AC
38	AB	AB	AB	AC	AB	ABC	AC	A	AB	AB	B	ABC	AB
39	AB	A	AB	A	AB	ABC	AC	A	AB	AB	AB	ABC	B
40	B	AB	ABC	AC	AB	ABC	AC	A	A	A	B	AC	B
41	B	B	AB	ABC	AB	ABC	AC	A	AB	AB	AB	ABC	A
42	B	AB	AB	ABC	B	A	AC	A	AB	AB	AB	AB	C
43	B	AB	AB	BC	AB	ABC	AC	AB	A	A	AB	ABC	A
44	B	AB	ABC	BC	B	ABC	A	A	A	AB	AB	ABC	AB
45	B	AB	ABC	BC	B	AB	AC	AB	A	AB	AB	AC	A
46	AB	AB	ABC	BC	AB	BC	AC	A	A	AB	B	AC	AB
47	AB	AB	ABC	BC	B	BC	A	A	A	AB	B	ABC	ABC
48	B	A	ABC	B	AB	BC	A	A	AB	A	AB	ABC	ABC
49	B	A	AC	BC	AB	C	AC	A	A	-	B	ABC	AB
50	B	A	AC	BC	AB	AB	C	A	AB	AB	B	ABC	AB
51	B	A	A	BC	AB	ACB	C	AB	AB	A	AB	AC	AB
52	B	AB	C	C	AB	B	AC	A	AB	AB	AB	A	AB
53	B	AB	A	AC	B	ABC	AC	AB	AB	A	B	A	AB
54	B	AB	A	AC	AB	ABC	A	AB	AB	AB	B	A	B
55	AB	A	ACB	ABC	AB	AB	AC	A	AB	AB	AB	A	B
56	AB	AB	ACB	ABC	AB	AC	ABC	AB	AB	AB	AB	A	AB
57	AB	A	ACB	ABC	AB	AB	C	A	AB	AB	B	A	AB
58	B	AB	ACB	BC	AB	ABC	A	AB	AB	AB	B	AB	AB
59	B	AB	ACB	ABC	AB	ABC	AC	AB	AB	AB	B	A	B
60	B	AB	AB	BC	AB	AC	ABC	AB	AB	AB	B	A	A
61	B	AB	B	AC	AB	AB	BC	AB	AB	AB	AB	AB	-
62	AB	AB	BC	AB	AB	ABC	BC	A	AB	AB	AB	AB	AB
63	AB	AB	BC	ABC	AB	ABC	BC	B	AB	AB	AB	AB	AB
64	AB	A	ABC	AC	AB	ABC	ABC	AB	AB	AB	AB	AB	AB
65	AB	A	AB	BC	B	AC	AB	AB	AB	AB	B	AC	AB
66	B	A	AB	BC	AB	AC	B	AB	AB	AB	B	B	AB
67	B	AB	AB	B	B	AC	B	AB	AB	AB	AB	C	AB
68	B	A	A+BC	AC	AB	AC	-	A	AB	AB	B	AB	A

Annexure II

DNA profile of individual genotypes of lentil for selected SSRs

Genotypes	SSR2 A:102 B:204	SSR4 A:103 B:273	SSR22 A:95 B:300	SSR23 A:80 B:135 C:195	SSR29 A:105 B:218	SSR32 A:90 B:330	SSR34 A:109 B:325	SSR39 A:70 B:216	SSR41 A:92 B:166	SSR53 A:113 B:102	SSR54 A:73 B:215	SSR55 A:100 B:300	SSR56 A:86 B:207	SSR60 A:80 B:98	SSR61 A:82 B:95	SSR71 A:85 B:220
1	A	A B	A	B	AB	B	A	AB	A	A	A	AB	B	A	B	AB
2	B	A B	A	A	AB	B	A	AB	A	B	AB	A	AB	A	A	A
3	B	A B	B	B	AB	AB	A	AB	B	B	AB	B	B	B	B	AB
4	B	A B	B	C	A	AB	A	A	B	B	AB	A	B	A	B	A
5	A, B	A	B	C	A	A	A	B	B	B	A	A	A	A	A	AB
6	B	A	B	A	AB	A	A	AB	A	B	A	AB	B	A	A	AB
7	B	A B	A B	AB	AB	B	A	AB	B	B	A	AB	B	B	A	AB
8	B	A B	A B	B	AB	B	A	B	B	A	A	AB	B	A	A	AB
9	B	A	A	A	A	A	A	A	A	A	B	A	A	A	A	AB
10	B	A	A	AC	A	A	A	AB	A	B	B	A	A	A	A	AB
11	A, B	A	A	AC	A	B	AB	AB	B	A	A	A	AB	A	A	B
12	B	A	A	AC	A	A	A	AB	A	B	AB	A	A	A	A	B
13	B	A	A	A	AB		AB	AB	A	B	AB	A	B	A	A	AB
14	B	A	A	C	A	A	A	A	A	B	B	A	A	A	A	AB
15	B	A	A	AC	A	B	A	AB	A	B	AB	A	B	A	A	A
16	A	A	A	A	AB	B	A	AB	A	B	AB	A	A	A	A	A
17	B	A	A	A	A	B	A	AB	A	B	AB	A	B	A	A	AB
18	B	A	A	A	A		A	A	B	B	B	A	AB	A	B	AB
19	B	A	A	A	A	AB	A	AB	A	B	A	A	AB	A	A	A
20	A	A	A	C	A	B	A	AB	A	B	A	A	A	B	A	B
21	A	A	A	C	A		A	A	A	B	B	A	AB	A	A	AB
22	A	A	A	C	A	A	A	AB	A	B	AB	A	B	A	A	B
23	A	A B	A	AB	AB	B	A	A	A	A	A	A	AB	A	A	B
24	A	A	A	AC	A	A	A	A	A	B	AB	B	AB	A	A	AB
25	A	A	A B	A	A	A	A	A	A	B	AB	A	AB	A	A	B
26	A	A	A	AC	A	B	A	A	A	B	A	A	A	B	A	AB
27	B	A	A B	ABC	AB	A	AB	A	A	A	B	A	A	A	A	B
28	A	A	A	C	A	B	AB	A	A	B	AB	A	AB	A	A	AB
29	B	A	A B	A	A	A	AB	A	A	B	B	A	A	A	A	AB
30	B	A	A	AC	AB	B	AB	A	A	B	AB	A	B	A	A	AB
31	B	A	A	ABC	A	B	AB	A	B	B	AB	A	AB	B	A	AB

n.b.: Size of the band is approximate value. A, B and C stands for the different size bands for a given primers

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