BIOLOGIA PLANTARUM 52 (4): 648-653, 2008

Assessment of genetic diversity of pigeonpea cultivars using RAPD analysis

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Abstract

In our present study assessment of genetic diversity and identification of pigeonpea cultivars has been done by employing 76 random amplified polymorphic DNA (RAPD) primers. Out of 796 amplified products, 587 showed polymorphism (73.7 %) and an average of 10.47 bands were amplified per primer. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped all the cultivars into three clusters. The cluster I consists of 7 cultivars, cluster II of 11 cultivars in 4 sub-clusters and cluster III 4 cultivars. Two cultivars were not included in any cluster. The clustering was strongly supported by high bootstrap values. Furthermore, high values of the average heterozygosity (H_{av}) and marker index (MI) also indicated the efficiency of RAPD as a marker system.

Additional key words: DNA fingerprinting, molecular marker, DNA amplification, *Cajanus cajan.*

Introduction

DNA markers, being independent of environmental interactions (*i.e*. highly heritable), unlimited in number and highly polymorphic, are considered to be the best tool for estimation of genetic diversity and development of an authentic fingerprint. In the present investigation, widely adapted, popular and high yielding cultivars of pigeonpea has been used. These genotypes possess the capacity to grow in the tropical and sub-tropical region across the globe. Most of the selected genotypes are originated from diversified pedigree; possess various geographical distribution; having different morphological character with varying maturity time and growth habit. RAPD, being a multi locus marker (Karp *et al*. 1997) with the simplest and fastest detection technology, has been used for diversity analysis in several crop plants including legumes (Weder 2002). Therefore, in our present study, we assayed the efficiency of the RAPD marker system to assay 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.

Materials and methods

Plants: All the twenty four *Cajanus cajan* (L.) Millsp. cultivars were collected from core collection maintained and grown in the breeders field by the respective pigeonpea breeder at Indian Institute of Pulses Research, Kanpur, India (Table 1).

DNA extraction: After collection of leaf samples from the 1-month-old seedlings of each genotype, isolation of DNA was done based on the modified protocol of Guillemant and Laurence (1992). 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 % polyvinyl-pyrrolidone; 1.4 % sodiumdodecyl sulphate), incubated at 65 °C for 30 min, added 0.6 volume of the 10 M ammonium acetate into each tube and kept for 15 min more at 65 °C followed by centrifugation at 11 180 *g* for 10 min. Supernatant was treated with 0.6 vol. of chilled iso-propyl alcohol, kept for 60 min at -20 °C, centrifuged, washed the pellet 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. DNA solution

Received 8 February 2007, *accepted* 15 November 2007.

Abbreviations: H_{av} - average heterozygosity; MI - marker index; RAPD - random amplified polymorphic DNA; UPGMA - unweighted pair group method using arithmetic averages.

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was further extracted twice with chloroform : iso-amyl alcohol (24:1) for further purification, re-precipitated in chilled ethanol and dissolved in TE buffer. Quality and quantity of purified DNA was checked by 0.8 % agarose gel electrophoresis using uncut lambda (λ) DNA as standard marker (300 μg cm^{-3}). Dilution of the DNA solution to 12.5 μg cm^{-3} was done for use in PCR analysis.

DNA amplification, documentation and data analysis: A total of 100 RAPD primers (OPAQ, OPAZ, OPX, OPH and OPP series of *Operon Technologies*, Alameda, USA) were screened and 76 primers that produced unambiguous polymorphic DNA profile was selected (Table 2). PCR mixture of 0.025 cm^3 contained 25 ng of genomic DNA template, 0.6 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 0.3 μM of decamer primer, 0.0025 cm^3 of 10× PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM MgCl2) and 0.025 cm^3 of pooled dNTPs (100 mM each of dATP, dCTP, dGTP and dTTP from *Fermentas Life Sciences*, Maryland, USA). PCR 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. and lastly primer extension at 72 °C for 7 min was provided. PCR products separation was done through 1.5 % agarose gel electrophoresis alongside *O'Gene Ruler*™ 100 bp DNA *Ladder Plus* (*Fermentas Life Sciences*) as molecular mass marker. The amplified products were documented under UV light source.

DNA bands were scored as 1/0 (presence/absence) and these binary data matrix was utilized to generate genetic similarity data using Jaccard's similarity coefficient. Unweighted pair group method using arithmetic averages (UPGMA) 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) through generating one thousand samples by re-sampling with replacement of characters with in the combined 1/0 data

Table 1. Brief information about pigeonpea cultivars used in the present study (SMD - Sterility Mosaic Disease; PSB - *Phytophthora* stem blight)

Genotypes with pedigree	Morphology with resistance	Genotypes with pedigree	Morphology with resistance
1) UPAS 120 Selection from P 4768	Semi-spreading, indeterminate, *Fusarium* resistant	13) ICPL 87119 C-11 × ICPL 6	Indeterminate, spreading, SMD and *Fusarium* resistant
2) MAL 6 MA-2 × Bahar	Spreading, SMD resistant	14) Pusa 9 UPAS 120 × 3673	Indeterminate, erect, SMD and *Alternaria* blight resistant
3) MAL 13 (MA-2 × MA 166) X Bahar	Spreading, SMD resistant	15) Pusa 992 Selection (ICRISAT line of ICPL 90306)	Semi-spreading, indeterminate, early maturing, *Fusarium* wilt resistant
4) PDA 10 Local Selection (Akbarpur/Kanpur Dehat; North India)	Compact, erect, indeterminate, large seeded, SMD resistant	16) CO 5 Mutant of CO 1	Semi-spreading, bushy, SMD resistant (moderately)
5) PDA 92-1 Bahar × ICP 8863	Spreading, indeterminate small seeded, SMD and *Fusarium* resistant	17) CO 6 Mutant of SA 1	Semi-spreading, indeterminate, pod borer tolerant
6) IPA 402 Local selection (Jaunpur; North India)	Semi-spreading, SMD resistant	18) BMSR 853 (ICP 7336 × BDN-1) × BDN-2	Spreading, large and white seeded, SMD and *Fusarium* wilt resistant
7) IPA 602 Bahar × ICPL 84023	Compact, erect, large seeded, SMD resistant	19) Amar Selection from Bahar	Indeterminate, erect, SMD resistant
8) IPA 3-1 Bahar × ICPL 96058 Selection (ICRISAT lines)	Compact, erect, SMD resistant	20) Bahar Selection (land race of Motihari; North-east India)	Indeterminate, compact, erect, large seeded, SMD resistant
9) IPA 3-2 Bahar × ICPL 96058 Selection (ICRISAT lines)	Compact, erect, SMD and *Fusarium* resistant	21) T-7 Selection (land race of Lucknow; North India)	Compact, erect, tall, large seeded, SMD and *Fusarium* wilt susceptible
10) ICPL 84023 Selection (ICRISAT lines)	Semi-spreading, determinate, SMD, *Fusarium* and PSB resistant	22) DA-11 Bahar × NP (WR) 15	Compact, erect, SMD and *Alternaria* blight resistant
11) ICPL 88039 Selection (ICRISAT lines)	Semi-spreading, early maturing, water logging tolerant, SMD resistant	23) NDA-1 Selection (land race of Faizabad; North India)	Indeterminate, compact, erect, SMD resistant, wilt tolerant
12) ICP 8863 Selection from land race (Maharashtra; Western India)	Spreading, indeterminate, medium seeded, *Fusarium* wilt resistant	24) KPL 43 Selection from Bahar	Indeterminate, compact, erect, SMD, *Fusarium* and PSB resistant

matrix. The expected heterozygosity for a genetic marker (H_n) was calculated by $H_n = 1 - pi^2$ (where pi is the allele frequency of the i^{th} allele; Nei 1987). The values of H_n was used to calculate H_{av} (the arithmetic mean heterozygosity) by the formula $H_{av} = \sum H_n/n$ (n = number of markers or loci analysed, Powell *et al.* 1996). The average heterozygosity for polymorphic markers $(H_{av})p$ was derived as $(H_{av})p = \sum H_n/np$ (np = number 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 $n\beta$ where β is the fraction of polymorphic marker or loci).

Results

Polymorphism and marker efficiency: Scorable 76 polymorphic RAPD primers led to amplification of 796 fragments ranging from 4700 bp (by OPP 14) to 250 bp (by OPAQ 18), out of which 587 (73.7 %) bands were polymorphic. The level of polymorphism ranged from 9.1 to 100 %. Maximum number of 21 amplified products was obtained by primer OPAQ 18 followed by 20 bands by OPAQ 19 and 19 bands each by OPP 10 and OPAQ 05. A minimum of 2 bands each was amplified by two primers (OPH 16, OPH 20). An average of 10.47 bands per primer was obtained and 33 primers (43.4 %) produced more than 10.47 bands. Among primer kits, a maximum of 19/20 primers responded in OPH, while it was 18/20 in case of OPP. Again, 80.3 % of bands out of a total of 208 amplified in OPP showed polymorphism followed by 77.1 % out of a total of 188 amplified in OPH (Fig. 1*a*,*b*). For marker efficiency, heterozygosity was calculated for all the 796 amplified products obtained across the cultivars. The H_{av} and $(H_{av})p$ were found to be 0.48 and 0.652, respectively, whereas the value of the marker index (MI) was 5.027.

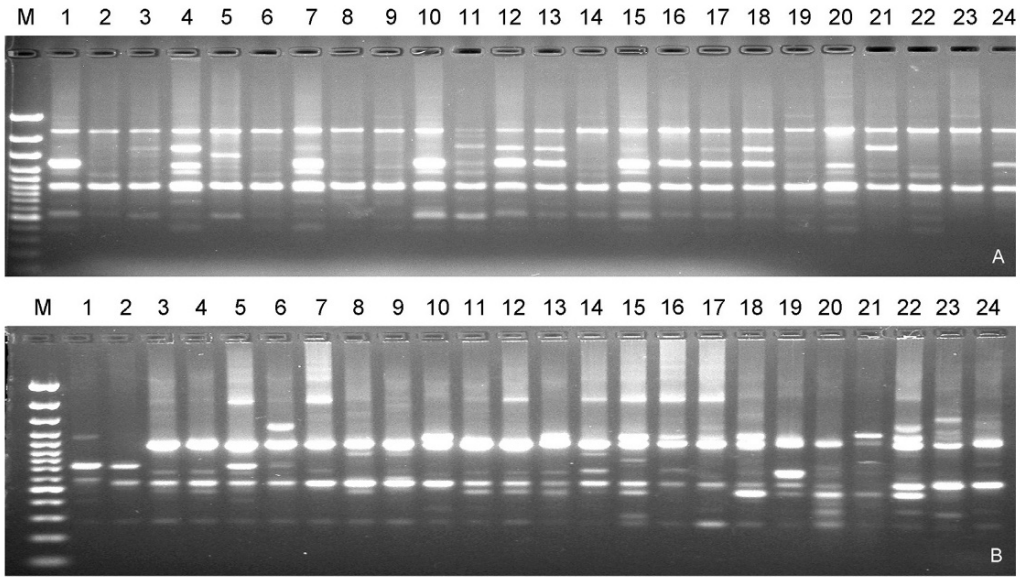

Fig. 1. RAPD profile of pigeonpea cultivars obtained with primers OPP 09 (*A*) and OPAZ 18 (*B*). Serial numbers of the cultivars correspond to Table 1. M - standard DNA marker, 100 bp DNA ladder plus.

Genetic relationship, genotyping and identification: The degree of genetic relatedness among pigeonpea genotypes varied considerably (0.567 to 0.827) due to the diversification in terms of morphology, maturity groups, parentages and geographical distribution. Highest similarity (0.827) was measured between IPA 602 and ICPL 84023. Few more pairs viz. Co5/Co6; PDA 10/IPA 602; IPA 3-1/IPA 3-2 and ICPL 84023/Pusa 992 were also found to show high degree of commonness. Least genetic similarity (0.567) was obtained between two pairs of cultivars. One pair consists of semi spreading, determinate ICPL 84023 and compact, erect, tall T7 where as the other pair is ICP 8863 (spreading, indeterminate) and T7. In general, T7 and NDA-1 was found to show considerable amount of diversity with few more genotypes. Multivariate analysis based upon the genetic similarity data grouped the cultivars into three major clusters (I, II and III). Cluster I consists of seven genotypes and four of them (PDA 10, IPA 602, ICPL 84023 and Pusa 992) formed the core cluster. Cluster II comprised of 4 sub-clusters (i, ii, iii and iv), with a total of 11 genotypes and cluster III consists of four genotypes. However, two cultivars (NDA-1 and T7), due to their considerable diversity with other genotypes 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. In the present investigation, a good number of distinct banding pattern pertaining to specific cultivar/breeding line was obtained. A total of 32 primers (42.1 %) produced 55 unique products with a range of 320 bp to 3100 bp (Table 2).

Table 2. Analysis of the unique DNA profiles obtained with 76 polymorphic RAPD primers in pigeonpea (serial number of genotypes are as given in the Table 1)

Genotype	Primer	[bp]	Genotype	Primer	[bp]	Genotype	Primer	[bp]
1	OPX 03	2000	12	OPAQ 19	2400, 2200,1400, 1300, 500	18	OPP 10	2000
	OPAZ 05	2200,1900		OPH 17	700	19	OPP 19	1100
	OPH 01	900		OPP 14	2000	20	OPP 08	320
4	OPX 04	800	14	OPAQ 20	400	21	OPAZ 03	700
5	OPH 11	2500	15	OPAZ 18	900		OPAZ 11	750
	OPP 09	1450		OPP 02	2600		OPH 10	2000
	OPP 10	2150, 1300		OPP 05	780		OPP 04	600
7	OPP 03	910	16	OPAQ 04	1350		OPP 05	800
8	OPH 17	1250	17	OPP 05	1200	22	OPH 03	800, 625
	OPP 04	520	18	OPX 04	700	23	OPX 12	1500
9	OPH 10	1150		OPAQ 05	1100, 1031,425		OPAZ 18	1500
10	OPAQ 09	500		OPH 01	1950		OPP 07	1400, 1200
11	OPP 04	500		OPH 12	1350, 1300	24	OPAQ 16	430
12	OPAQ 18	3100, 2500,425		OPP 06	550		OPAZ 16	900

Discussion

In our study, an attempt was made to examine the extent of genetic variation present in the popular cultivated pigeonpea genotypes as well as their precise identification through efficiency of polymorphic RAPD primers.

RAPD is an effective tool to evaluate and reveal

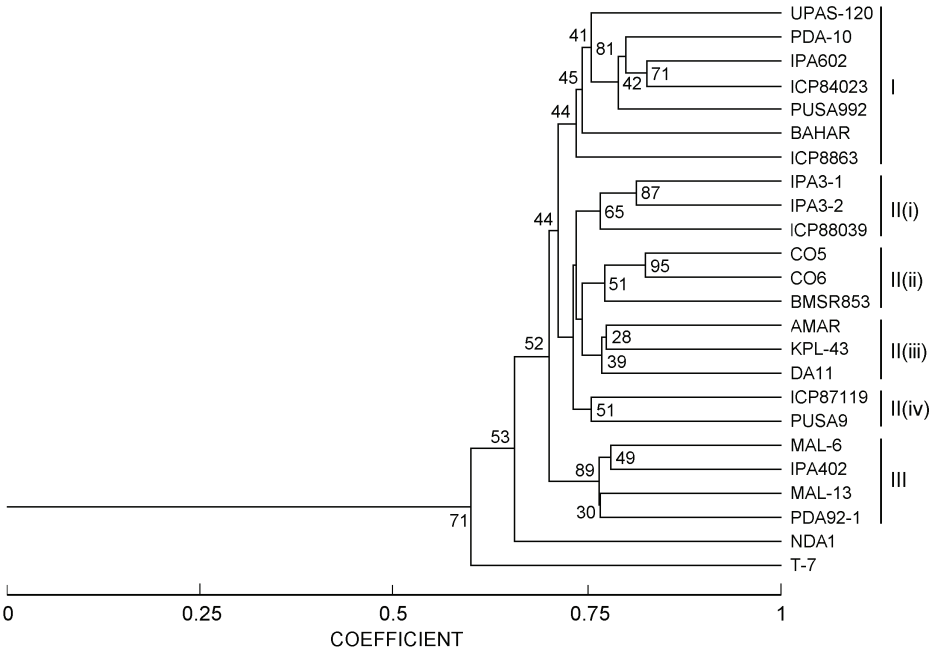

Fig. 2. Dendrogram of pigeonpea varieties constructed using UPGMA based on 76 RAPD primers. The major clusters and sub-clusters are indicated on right margin. Numbers at branch points indicate support for varieties clustered; values are percent of bootstrap sample that exhibited the cluster (no number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

molecular diversity not only in pulses like chickpea (Ahmad 1999), field pea (Simioniuc *et al.* 2002, Tar'an *et al.* 2005), mungbean (Lakhanpaul *et al*. 2000), *etc*., but also in tuber crop like potato (Chakrabarty *et al*. 2006) and cereals like rice (Ray Choudhury *et al*. 2001), wheat (Cao *et al*. 2000) and maize (Pejić *et al*. 1998). In our study, 73.7 % fragments were found to be polymorphic with an average of 10.47 bands per primer and is well comparable with the results obtained by Ratnaparkhe *et al.* (1995), where 7.93 bands per primer was obtained using 16 polymorphic RAPD primers in 10 pigeonpea cultivars. Moreover, estimated genetic similarity obtained by the same workers varied from 0.7 to 0.9, whereas in our studies the range widened to 0.567 to 0.827 because of the high variability among the selected genotypes. RAPD has been found to be well correlated with other marker systems. In AFLP studies, diversity was found within a narrow range of 0.82 - 1.00 with little polymorphism of 13.28 % in 20 pigeonpea cultivars (Panguluri *et al*. 2006), thus indicating RAPD as an efficient marker system. In another study of field pea genetic diversity (Baranger *et al*. 2004), the mean allelic frequency was found highest for RAPD over other markers and the structure of genetic variability by RAPD was very close to that obtained with other marker systems.

Quantitative estimation of marker utility and detection of polymorphism is depicted in terms of mean heterozygosity and marker index (Powell *et al*. 1996). Polymorphism within a population is detected by the number of alleles present at a locus and their frequency of distribution, whereas heterozygosity is the probability that two alleles taken at random from a population can be distinguished using a marker system (Dangi *et al*. 2004). The mean heterozygosity (H_{av}) using allozyme diversity was found less in self pollinating legumes like cowpea (0.027; Pasquet 2002) and wild lentil (0.342; Huh and Huh 2001). In RAPD, the H_{av} value was found to be 0.203 and 0.346 in two different *Trigonella* species (Dangi *et al*. 2004). In our study however, the H_{av} was found to be 0.652 and the marker index (MI) was obtained to be 5.027, thus proved the efficiency of polymorphic RAPDs as a marker system in detecting heterozygosity in often cross pollinated species like pigeon pea.

Estimation of variability in pigeonpea using protein and isozymes (Ladizinsky and Hamel 1980, Kollipara *et al*. 1994) was not much successful due to limited polymorphism. Present study developed altogether more number of bands with higher polymorphism than the earlier AFLP and RAPD analysis conducted on pigeonpea. The average similarity index of 75.0 between cultivars along with the average number of bands developed per primer (10.47) and the average percent polymorphism (73.7) indicated the efficiency of polymorphic RAPD primers chosen for the study. Highest similarity (0.827) between the pair of cultivars was due to the common parentage of ICPL 84023 where as least similarities were obtained between two pairs where T7 is a common genotype. Further, NDA 1 (compact, erect and intermediate land race from northern India) also showed significant variations with two ICRISAT genotypes ICPL 87119 (spreading, intermediate) and ICPL 84023 (semi-spreading, determinate).

The clusters of the dendrogram and its robustness analyzed by the bootstrap supported the ability of RAPD to represent the genetic structure of the collection. The genotypes in the cluster I are of intermediate growth habit and many of them are indeterminate. They are selection from land races of northern and south-western part of India. Potential of most of these germplasms has been identified at ICRISAT. For instance, UPAS 120 (selection from ICP 3337 and has got ICRISAT accession number of P 4768) and other four genotypes in this cluster has been developed from lines identified at ICRISAT. Out of the three genotypes of the sub-cluster II (i), IPA 3-1 and IPA 3-2 are selections from Bahar × 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 and tolerant lines for water logging conditions. All the three cultivars of the sub-cluster II (ii) with semi-spreading nature are being grown in the same agro-climatic and geographical location. Three erect and compact cultivars of sub-cluster II (iii) Amar, KPL 43 and DA-11 are of long duration, habitat of north-east plain zone of India and have one common parent Bahar. Both the sterility mosaic disease resistant cultivars of sub-cluster II (iv) *i.e*. ICPL 87119 and Pusa 9 have been developed through hybridization and are of indeterminate growth habit with semi-spreading to compact plant type. The spreading or semi-spreading cultivars form the cluster III and are of long duration maturity group. Bahar, a land race from north-east India is the common parent of MAL 6, MAL 13 and PDA 92-1, whereas the remaining genotype IPA 402 is again a land race and habitat of north-eastern part of India. Two cultivars NDA 1 and T7 with different morphological parameters could not be included in any cluster and originated from diverse land races of northern India. Both of them are compact and erect in nature and could be used extensively for breeding programme because of their diversification from other cultivars and high yield potential.

Appropriate identification of crop cultivars is pre-requisite for detection of duplicates, cultivar registration and protection of plant breeders' right. For defining DUS (distinctiveness, uniformity and stability), DNA data is well accepted along with the morphological data. In the present study, creation of basic data set using polymorphic RAPDs has been done. Moreover, 55 unique products across cultivars (Table 2), could be used as ready reference for cultivar identification and could also be converted into CAPS or SCAR marker for cultivar confirmatory tests.

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