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Identification of genotype specific markers and assessment of genetic relatedness among pigeonpea cultivars using RAPD

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ABSTRACT

Twenty-one polymorphic RAPD primers providing proper amplification pattern were selected for identification of genotype specific markers and assessment of genetic relatedness among pigeonpea cultivars. Among these, 16 were found to be unique producing 40 genotype specific bands in 16 different genotypes. An average of 12.6 bands per primer was obtained with 89.4% polymorphism. Clustering based on Jaccard's similarity coefficient using UPGMA grouped all the cultivars into two major clusters and clustering was supported by high bootstrap values. The arithmetic mean heterozygosity (H_{av}), the average heterozygosity for polymorphic markers (H_{av})p and marker index (MI) were calculated and found high, which indicated the correctness of the generated profile and efficacy of RAPD as a marker system. The genotype specific bands developed by the RAPD primers could be used for cultivar identification.

Key words: *Cajanus cajan*, Genetic relationship, RAPD

Pigeonpea (*Cajanus cajan* L. Millsp.) is one of the most important pulse crops in the arid and semi-arid tropics. It is mostly a rainfed crop and due to its hardy nature, it is very popular among small and marginal farmers. In India, pigeonpea occupies the second most important position among pulses. In order to improve production, a large number of varieties have been released for cultivation in different agro-climatic conditions. To identify unique primers that can identify specific genotypes and determine the genetic diversity have emerged as important aspect of crop improvement programmes. As evident from available informations, there is limited molecular data towards characterization of pigeonpea cultivars. RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeats), microsatellites and AFLP (Amplified Fragment Length Polymorphism) are the commonly used techniques for molecular characterization of genotypes. RAPD is not only the fastest, simplest and least costly, but a single primer can identify multiple loci in a single reaction (Karp *et al.* 1997). Molecular diversity analysis using RAPD has not only been used in pulses like chickpea (Ahmad 1999), mungbean (Lakhanpaul *et al.* 2000) and pea (Taran *et al.* 2005) but also in cereals like rice (Ray Choudhury *et al.* 2001), wheat (Cao *et al.* 2000) and in many other versatile crops like potato (Chakrabarti *et al.* 2006), mango (Ravishankar *et al.* 2000) and roses (Jan *et al.* 1999). Among the very few reports of RAPD as a marker in pigeonpea, identification of CMS lines (Souframanien *et al.* 2003) is a classical example.

MATERIALS AND METHODS

Pigeonpea cultivars from different maturity groups were collected from the collections maintained at Indian Institute of Pulses Research, Kanpur (Table 1). For DNA extraction, a modified protocol without liquid nitrogen was used (Guillemant

Table 1. Details of pigeonpea cultivars used in the present study

Cultivar	Pedigree	Character
UPAS 120	Selection from P 4768	Semi spreading and indeterminate, (NEPZ, NWPZ)
PDA 10	Local Selection from Akbarpur, Kanpur Dehat, U.P.	Compact, erect and indeterminate; (NEPZ)
IPA 602	Bahar x ICPL 84023	Erect and compact; (NEPZ)
ICPL 84023	Selection from ICRISAT germplasm lines	Semi spreading and determinate; (SZ)
Pusa 992	Selection from ICPL 90306	Indeterminate and semi spreading; (NWPZ)
Bahar	Selection from land race of Motihari, Bihar	Indeterminate, compact and erect; (NEPZ)
MAL 6	MA 2 X Bahar	Spreading; (NEPZ, CZ)
MAL 13	(MA 2 X MA 166) X Bahar	Spreading, (NEPZ)
PDA 92-1	Bahar x ICP 8863	Spreading and indeterminate, (NEPZ)
IPA 402	Local selection from Jaunpur, U.P.	Semi Spreading; (NEPZ)
IPA 3-1	Bahar X ICPL 96058	Erect and compact; (NEPZ)
Amar	Selection from Bahar	Indeterminate, erect, (NEPZ)
Co 5	Mutant of Co 1	Semi spreading; (SZ)
Co 6	Mutant of SA 1	Indeterminate, semi spreading, (SZ)
BSMR 853	(ICP 7336 X BDN 1) X BDN 2	Spreading; (CZ)
KPL 43	Selection from Bahar	Indeterminate, erect and compact; (NEPZ)
DA 11	Bahar x NP (WR) 15	Erect and compact; (NEPZ)
Pusa 9	UPAS 120 X 3673	Indeterminate and erect, (NEPZ)
IPA 3-2	Bahar X ICPL 96058	Erect and compact; (NEPZ)
ICPL 88039	Selection from ICRISAT germplasm lines	Semi spreading; (SZ)
ICPL 87119	C 11 X ICPL 6	Indeterminate and semi spreading; (SZ, CZ)
ICP 8863	Selection from land race of Maharashtra	Spreading and indeterminate; (SZ, CZ)
NDA 1	Selection from land race of Faizabad, U.P.	Indeterminate, erect and compact; (NEPZ)
T 7	Selection from land race of Lucknow, U.P.	Erect and compact; (NEPZ)

(NEPZ= North-East Plain Zone, NWPZ= North-West Plain Zone, SZ=South Zone, CZ= Central Zone)

and Laurence 1992). Leaves from young seedlings were pulled together and ground with grinding buffer until a thick paste was formed. The composition of the grinding buffer was as follows: 100 mM sodium acetate, pH 4.8; 500 mM NaCl; 50 mM EDTA, pH 8.0; 50 mM Tris, pH 8.0; 2% PVP; 1.4% SDS. Purification of DNA was done twice with extraction of Phenol: Chloroform: isoamyl alcohol (25:24:1). RNAse (@ 40µl from 1mg/ml stock was applied in the supernatant to get rid of RNA. DNA quality and quantity from all the individual cultivars were checked through 0.8% agarose electrophoresis with standard DNA before PCR amplification. A total of 21 random primers (Operon Technologies, Alameda, CA, USA) were selected for PCR analysis based upon their performance and reproducibility already tested in our laboratory. PCR mixture of 25 µl contained 25 ng of genomic DNA template, 0.6 µq 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 condition used for RAPD amplification 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. The amplified products as developed by the primers were separated by agarose (1.5%) gel electrophoresis and documented in gel documentation system (BioRad XR, Biorad, USA). O'Gene Ruler™ 100 bp DNA Ladder Plus (ladder range 3000 bp to 100 bp from Fermentas Life Sciences, USA) was used as molecular weight marker. DNA bands were scored for its presence/absence (1/0) for each primer genotype combination. Software NTSYS-pc, version 1.7 (Rohlf 1992) was used for estimation of genetic relatedness between the cultivars using Jaccard's similarity coefficient and clustering was done with UPGMA (unweighted pair group method using arithmetic averages). Strength of clusters was evaluated by bootstrap analysis using Win Boot software (Yap and Nelson 1995). The arithmetic mean heterozygosity for a marker (H_{av}) was calculated by $H_{av} = H_n/n$ [n = number of markers or loci analysed (Powell *et al.* 1996); Heterozygosity for a marker (H_n) = $1\text{-}pi^2$ where pi is the allele frequency of i^{th} allele (Nei 1987)]. The average heterozygosity for polymorphic markers (H_{av})p was derived by $\delta H_n/np$ (np = no. of polymorphic markers or loci). Marker index (MI) was also calculated as MI = E (H_{av})p [E (effective multiplex ratio) = nâ, â is the fraction of polymorphic marker or loci].

Table 2. Genotypes and their unique amplified products identified by RAPD primers

Cultivar	No. of unique band(s)	Primer name	Molecular weight (bp)
UPAS 120	5	OPBB 14	2250, 2000, 1550, 1500, 1350
MAL 6	1	OPBB 07	650
MAL 13	1	OPBB 16	2500
IPA 602	1	OPP 03	910
IPA 3-1	1	OPH 17	1250
IPA 3-1	1	OPP 04	520
IPA 3-2	1	OPH10	1150
ICPL 84023	5	OPBB 16	950, 800, 750, 500, 425
ICPL 88039	1	OPBB 07	750
ICP 8863	3	OPAQ 18	3100, 2500, 425
ICP 8863	5	OPAQ 19	2400, 2200, 1400, 1300, 500
ICP 8863	1	OPH 17	700
ICP 8863	1	OPBB 16	1050
Pusa 9	1	OPAQ 20	400
Pusa 992	1	OPBB 13	3000
Amar	1	OPP 19	1100
Bahar	1	OPP 08	350
T 7	1	OPH 10	2000
T 7	1	OPP 04	600
DA 11	2	OPH 03	800, 625
DA 11	1	OPBB 04	3000
DA 11	1	OPBB 13	1300
NDA 1	2	OPP 07	1400, 1200
NDA 1	1	OPBB 16	1375

Table 3. Amplified products and polymorphism obtained with RAPD primers

Primer	No. of amplified band	Polymorphic band	Monomorphic band	Percentage polymorphism
OPAQ 18	21	20	1	95
OPAQ 19	20	19	1	95
OPAQ 20	18	14	4	77.7
OPAZ 03	7	6	1	85.7
OPAZ 19	7	7	0	100
OPH02	10	9	1	90
OPH 03	14	12	2	85.7
OPH 05	8	5	3	62.5
OPH 10	11	10	1	90.9
OPH 14	9	8	1	88.8
OPH 17	12	9	3	75
OPP 03	9	9	0	100
OPP 04	15	15	0	100
OPP 07	11	9	2	81.8
OPP 08	17	15	2	88.2
OPP 19	7	5	2	71.4
OPBB 4	12	8	4	66.6
OPBB7	15	15	0	100
OPBB13	10	10	0	100
OPBB14	13	13	0	100
OPBB16	19	19	0	100

RESULTS AND DISCUSSION

In the present study, out of a total of 40 primers tested, 21 RAPD primers showing polymorphism and providing proper amplification pattern were selected. Out of these primers, 16 showed uniqueness by producing 40 genotype specific bands in 16 different genotypes (Table 2). A total of 265 amplified products were obtained with an average of 12.62 bands per primer (Table 3), out of which 237 (89.4%) bands were polymorphic with an average of 11.29 bands per primer. DNA amplification pattern as detected by two of the RAPD primers used in the present study (OPP 04 and OPBB 16) have been provided in Fig. 1a,b. Molecular diversity analysis vis-à-vis identification of cultivars is important for variety registration, protection of plant breeders' right and identification of quite similar/duplicate genotypes. Moreover, along with the morphological data, DNA data is well accepted in defining DUS (distinctiveness, uniformity and stability). In

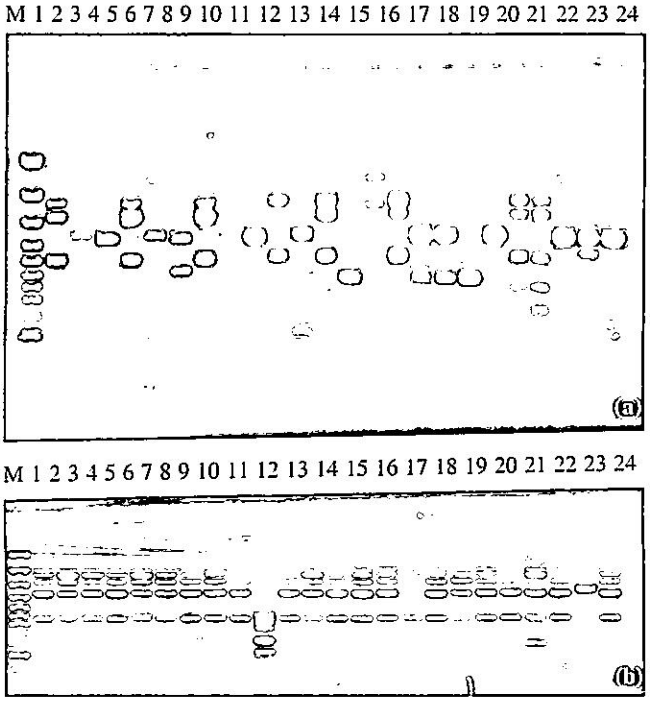

Fig. 1(a, b). RAPD profile of pigeonpea cultivars obtained with primers OPP 04 (a) and OPBB 16 (b). Serial number of the varieties are as follows: UPAS 120, MAL 6, MAL 13, PDA 10, PDA 92-1, IPA 402, IPA 602, IPA 3-1, IPA 3-2, ICPL 84023, ICPL 88039, ICP 8863, ICPL 87119, Pusa 9, Pusa 992, Co 5, Co 6, BSMR 853, Amar, Bahar, T 7, DA 11, NDA 1 and KPL 43. M=Standard DNA marker, 100 bp DNA ladder

this study, genotype specific DNA amplification products could be used as reference for fingerprint and in due course these bands could be converted into CAPS or SCAR marker for varietal confirmatory tests. For diversity studies in pigeonpea, biochemical markers were not found suitable due to little polymorphism obtained (Kollipara *et al.* 1994). RAPD, however, has been successfully employed in many legumes and other crops for molecular diversity as stated earlier. In the present study, the percent polymorphic bands as detected by RAPD primers (89.4%) has been found much higher as compared to AFLP, where polymorphism obtained was only 13.3% (Panguluri *et al.* 2006).

Efficiency of markers and their utility in terms of polymorphism and quantitative estimation could be expressed in mean heterozygosity and marker index. The H_{av}, $(H_{av})p$ and MI were found to be 0.578, 0.647 and 8.16, respectively. When these values were compared with values from other crops using RAPD as a marker system, the H_{av} value was found to be 0.20 and 0.35 in two *Trigonella* species (Dangi *et al.* 2004), whereas in field pea cultivars H_{av} and $(H_{av})p$ were found to be 0.496 and 0.663, and the MI value was 4.787 (Ray Choudhury *et al.* 2007). In the present study, all the values were found to be higher, thus proving the evidence of correct choice of primers and usefulness of RAPD as a marker system in detecting diversity in pigeonpea.

Similarity coefficient data among pairs of pigeonpea cultivars showed sufficient variability (0.455 to 0.828, average of 0.71) as compared to 0.7 to 0.9 obtained in earlier studies in pigeonpea (Ratnaparkhe *et al.* 1995). The pair of genotypes which indicated maximum similarity was Co 6 and BMSR 853 (0.828) and Co 5 and Co 6 (0.827). Other pairs of genotypes showing high degree of similarity (>0.8) were Co 5/BMSR 853, MAL 6/MAL 13, IPA 602/PDA 10, and IPA 3-1/Amar. Least similarity (0.455) was found between ICP 88039 and T 7. UPAS 120 and T 7 showed considerable amount of diversity with many genotypes. NDA 1 was also found to show high diversity with most of the genotypes. Cluster analysis using UPGMA grouped the cultivars into two major clusters (I and II, Fig. 2) with 6 and 15 genotypes, respectively. Cluster I consisted of six genotypes and they are of intermediate growth habit and many of them are indeterminate. These cultivars are developed through selection from land races of north and south-western India and mostly grown in high rainfall areas of north-east plain zone and south zone. Bahar and ICP 84023 are parents of IPA 602. PDA 10 is a selection from the land race, wheras Pusa 992 and UPAS 120 both are selections from the ICP germplasm lines of ICRISAT IPA 402. Cluster II was divided into three sub-clusters (A,B,C) and the sub-cluster IIB was further sub-divided into 3 sub-groups (IIBi, IIBii and IIBiii). Sub-cluster IIA consisted of 4 genotypes and three of them (MAL 6, MAL 13 and PDA 92-1) have one common parent 'Bahar'. Another genotype in this cluster *i.e.*, IPA 402 and Bahar are

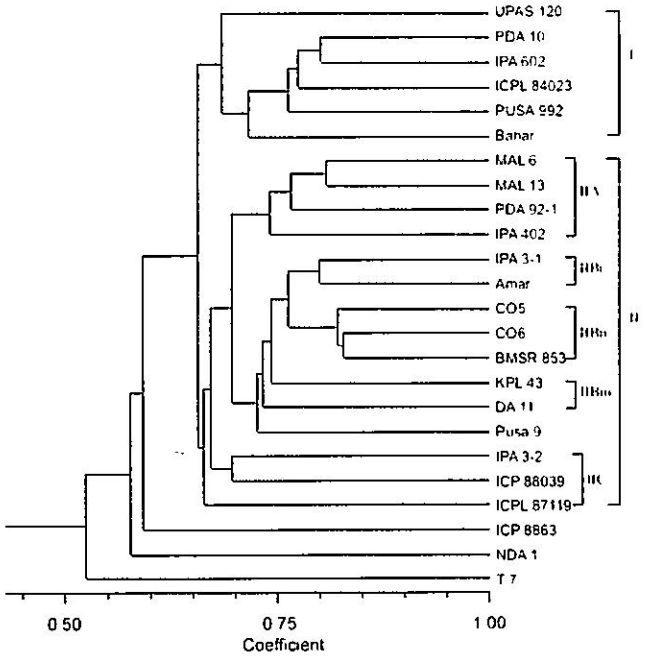

Fig 2. Dendrogram of pigeonpea cultivars constructed using UPGMA. The major clusters and sub-clusters are indicated on right margin. Number indicates the bootstrap value of corresponding clusters

local collections from farmer's field. All of them belong to long duration group, possess indeterminate growth habit with spreading or semi-spreading nature and are grown in north-east plain zone of India. Two erect type cultivars IPA 3-1 and Amar in the sub-group IIBi have Bahar as a common parent and are grown in the north-east plain zone of India. All the semi-spreading/spreading genotypes *viz.*, Co 5, Co 6 and BMSR 853 in the sub-group IIBii are from south and south-western part of India. Co 5 and Co 6 both are mutants of local landraces and BSMR 853 is also a progeny of local land race from south-west India. Two genotypes KPL 43 and DA 11 in the sub-group IIBiii have Bahar as a common parent and all the three genotypes are erect type. The sub-cluster IIC possessed three genotypes with parentages from ICRISAT germplasm lines. Three cultivars NDA 1, T 7 and ICP 8863 showed considerable diversity and could not be included in any cluster. All the clusters and sub-clusters in the dendrogram were supported by high bootstrap values, thus again indicating that the RAPD could be a good choice to classify the genotypes. Genotypes with high molecular diversity could be the choice for breeding experiments and development of gene pools with broad genetic base.

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