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Study of genetic diversity in mungbean (*Vigna radiata* L. Wilczek) cultivars using RAPD markers

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Abstract
A set of twelve cultivars of mungbean [*Vigna radiata* (L.) Wilczek] were taken as experimental materials in the present study. The young leaves were collected from five individual plants and leaf samples were pooled for each accession. Random amplified polymorphic DNA (RAPD) markers were used to study the DNA polymorphism in twelve Indian mungbean cultivars. A total of 17 random primers were used in the research and 11 of them generated reproducible RAPD patterns. Out of 17 random primers, a total of 11 highly polymorphic primers were used for amplification of genomic DNA isolated from 12 mungbean cultivars in this study. Thus the result is based on the amplification products of 11 polymorphic primers with 12 cultivars of mungbean. Amplification of genomic DNA of 12 Indian mungbean cultivars with 11 RAPD primers yielded 152 fragments that could be scored, of which 108 were polymorphic, thus with average number of bands amplified per primer was 13.8 and with an average of 9.8 polymorphic fragments per primer. Number of amplified fragments with random primers ranged from 8 (OPA 14) to 22 (OPA11). Percentage polymorphism ranged from 23.08% (OPA09) to a maximum of 100% (OPN 05 and OPN 016), with an average of 71.05%. The Jaccard's similarity coefficients matrix based on RAPD profiles were subjected to UPGMA cluster analysis. The RAPD cluster pattern segregated the 12 mung bean cultivars into two major clusters. The cultivar Pusa-0672 formed Cluster 1 (C-1) and cultivars Samrat, BPMR-145, HUM-2, MUM-2, Pairy Mung, Pant M-2, Pant M-4, PKV AKM-4, Pusa-9072, RMG-62 and RMG-268 have made Cluster 2. This indicated the narrow genetic base in the Indian mung bean cultivars used in the study.

Keywords: RAPD, genomic DNA, polymorphism, genetic diversity and mung bean

Introduction
Mung bean (green gram, *Vigna radiata* (L.) Wilczek) is an Asiatic species with a considerable importance as it is a widely cultivated pulse crop, because of its adaptation to short growth duration, low water requirement, soil fertility and because it can be used in crop rotation practices also. In India and some South Asian countries, it contributes significant dietary protein supply in predominantly cereal rich diets. Its contain 25.9 % protein and 504 mg/g lysine content and it is most useful in vegetarian diet. The yield of mungbean has not been increased substantially due to insufficient use of genetic diversity in breeding programmes (Bernatzky and Tanksley 1989) [1]. The productivity of pulses is very low as compared to cereals, which have been selected for high grain yield under high input conditions (Narasimhan *et al.* 2010) [10]. The major constraints in achieving high yield of this crop are lack of genetic variability, poor harvesting index and susceptibility to diseases and pests. Despite the efforts, development of sustainable resistant cultivar with higher yields has not yet been successful due to narrow genetic bases of the present cultivars.

The assessment of genetic diversity is a prerequisite and important step for the improvement of any crop plant. The estimation of genetic diversity is invaluable in selection of diverse parental combinations to generate segregating progenies with maximum genetic variability and introgressing desirable traits from diverse or wild germplasm into the cultivars to broaden the genetic base. Earlier, genetic diversity studies were mostly carried out based on morphological characters and isozyme markers. Limited availability, low polymorphism and high influence of environmental factors on the expression, limited the use of morphological and biochemical markers. Molecular markers provide an alternative and important tool for genetic analysis as they are numerous, selectively neutral and allow screening at any growth stage (Soller and Beckmann, 1983) [16].

The available germplasm serves as the most valuable natural reservoir for providing the required plant attributes for obtaining the high yielding crop varieties (Hawkes, 1981) [5]. In order to utilize such accessions with maximum required plant attributes, it is necessary to screen and characterize the germplasm for the nature and extent of genetic diversity included in it. Characterization and cataloguing of germplasm have been traditionally carried out using morpho-agronomic traits while in the last two decades or so the molecular markers have also been used for germplasm characterization. The molecular markers, highly heritable and available in large numbers are often polymorphic enough to enable discrimination of closely related genotypes. Among the several molecular methods possible, using Random Amplified Polymorphic DNA (RAPD) profiles (Williams *et al.*, 1990) [18] offers a rapid and reliable identification and characterization of genotypes by generating markers for comparative analysis that are quick, easy to use, free from environmental influences, unlimited in number, random but wide coverage of genome and have a relatively higher level of polymorphism (Newbury and Ford-Lloyd, 1993) [11]. Among the DNA markers, development of RAPD-PCR based DNA finger printing is easier (Gherardi *et al.* 1998) [4]. The objective of the present study was to investigate and compare genetic diversity using random amplified polymorphic DNA (RAPD) markers, for assessing the genetic base of released Indian cultivars of mungbean.

Material and Methods

Plant materials: A set of twelve cultivars of mungbean [*Vigna radiata* (L.) Wilczek] were taken as experimental materials in the present study. The mungbean seeds were obtained from Indian Institute of Pulses Research (ICAR), Kalyanpur, Kanpur (U. P.) for sowing during Kharif season, 2013-14. The names of cultivars obtained, pedigree and their source are given in Table 1.

Table 1: Name of Cultivars of Mungbean along with their Source and Pedigree

S. No	Name of cultivar	Pedigree	Source/Institution where developed
1.	SAMRAT	ML 20/19 x ML5	IIPR, Kanpur
2.	BPMR-145	JL-781 x Mungi	MKV, Parbhani
3.	HUM-2	Selection from local germplasm No.TVCM 3	BHU,Varanasi
4.	MUM-2	Mutant of K851	Meerut University, Meerut
5.	PAIRY MUNG	TARM-1 x J-781	Jointly evolved by BARC, Trombay and IGKV, Raipur
6.	PANT M-2	Mutant of ML-26	GBPAU, Pantnagar
7.	PANT M-4	T-44 x UPU-2	GBPAU, Pantnagar
8.	PKV AKM-4	BM-4x PS -7	PDKV, Akola
9.	PUSA-0672	11/395x ML267	IARI, New Delhi
11.	RMG-62	R-288-8xChina mung	RAU, Durgapura
12.	RMG-268	R-288-8x J -781	RAU, Durgapura

DNA Extraction and PCR assay: The young leaves were collected from five individual plants, and leaf samples were pooled for each accession. For genomic DNA isolation, 2 g fresh leaf tissue was ground in liquid nitrogen and DNA was isolated following the CTAB method of Doyle and Doyle (1990) [3] with minor modifications.

Prior to extraction of DNA, the pestle and mortar, spatula and scissors were sterilized by autoclaving process. 2 g leaf samples were taken and frozen in liquid nitrogen for making a fine powder. The fine powder was allowed to thaw in the presence of 500 µl of pre-heated CTAB extraction buffer containing β mercaptoethanol, in polypropylene centrifuge tubes and incubated in water bath for 45 min at 65°C with occasional mixing. The tubes were removed from the water bath and allowed to cool at room temperature and equal volume of chloroform and isoamyl alcohol mixture (24:1) was added and mixed by inversion for 15-20 min. It was then centrifuged at 10000 rpm for 15 min at room temperature. The clear aqueous phase/supernatant was transferred to a new sterile eppendorf tube. In supernatant, of 1/10 volume of 3M Sodium acetate (pH 5.2) and equal volume of ice-cold isopropanol were added and mixed gently by inversion until DNA was precipitated out and incubated at 4^0C for overnight. Then, eppendorf tubes were centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was discarded and DNA pellet was washed with 70% ethanol. The DNA pellets were air dried after washing with 70% ethanol. The DNA pellet was dissolved in 100 µl of TE buffer (pH 8.0) and stored at 4^0C.

Assay of DNA quality and quantity: The genomic DNA quality and quantity was estimated through gel electrophoresis and spectrophotometric measurement, respectively.

Gel analysis: Agarose gel (0.8%) was casted in 1X TAE (Tris Acetate EDTA) buffer containing ethidium bromide (0.5µg/ml) and then 10 µl of DNA sample mixed with 5 µl 6x loading dye was loaded in each well. Gel was run at constant voltage (50 V) for three hours. Gel was then visualized on gel documentation system.

Quantification of extracted DNA: To calibrate the spectrophotometer at 260 nm as well as 280 nm wave length, 1ml TE buffer was taken in a cuvette. Added 2 to 5 µl of DNA mixed properly and recorded the optical density (O.D.) at both 260 and 280 nm. Estimated the DNA concentration employing the following formula –

$$\text{Amount of DNA } (\mu g/\mu l) = \frac{A_{260} \text{ x } 50 \text{ x dilution factor}}{1000}$$

Selection of RAPD primers: A total of 17 random decamer primers (Operon Technologies, USA) were selected from Operon series for DNA amplification of 12 cultivars of mungbean. The G+C contents of the primers ranged between 50 to 80%. They were procured as lyophilised salts having concentrations of 200 µM to 1.0 mM. The experiment was conducted in two phases; in the first phase, 17 random decamer primers were used to optimize the annealing temperature of each primer and then amplified with respective optimizes annealing temperature. Out of 17 random primers, a total of 11 highly polymorphic primers (Table-2) were used

for amplification of genomic DNA isolated from 12 mungbean cultivars in this study. Thus the result is based on the amplification products of 11 polymorphic primers with 12 cultivars of mungbean.

Table 2: RAPD Primers used for DNA Finger Printing in Mungbean Cultivars

S. No.	Primer	Sequence(5´-3´)	Annealing Temperature ºC
1	OPA-02	5´-TGCCGAGCTG-3´	38
2	OPA-09	5´-GGGTAACGCC-3´	36
3	OPA-11	5´-CAATCGCCGT-3´	38
4	OPA-14	5´-TCTGTGCTGG-3´	36
5	OPN-05	5´-ACTGAACGCC-3´	38
6	OPN-08	5´-ACCTCAGCTC-3´	38
7	OPN-10	5´-ACAACTGGGG-3´	38
8	OPN-13	5´-AGCGTCACTC-3´	36
9	OPN-14	5´-TCGTGCGGGT-3´	38
10	OPN-15	5´-CAGCGACTGT-3´	38
11	OPN-16	5´-AAGCGACCTG-3´	38

PCR Amplifications: The PCR amplification was carried out according to the protocol of (William *et al.* 1990) [18] with minor modifications. Different temperature regime and concentration of components were used to determine optimised PCR protocol for RAPD markers assay. The parameters like annealing temperature, Mg^{++} concentration were optimized. After standardization, the polymerase chain reaction was carried out in a 25 µl reaction volume containing 30 ng of template genomic DNA, 1.5 mM PCR buffer (MBI Fermentas, USA), 400 µM dNTPs (MBI Fermentas), 1.5 units of *Taq* DNA polymerase (MBI Fermentas), and 4 µM of primer using a thermal cycler (T1, Biometra, Germany). The initial denaturation at 94 ºC for 4 min, followed by optimized consecutive cycles (40 cycles) of 1 min. at 94 ºC, 1 min. at optimized Ta and 1 min. at 72 ºC; the final extension was allowed for 10 min. at 72 ºC. After completion of PCR amplification, the samples were kept at -20 ºC until gel electrophoresis.

Resolution of the amplified DNA fragments: The amplified products were resolved by electrophoresis at 75 V (corresponding to 45 mA current) for 2 hrs. in 1.5% agarose gel in 0.5x TBE buffer. Stock ethidium bromide (10 mg/ml) solution was added to it to a final concentration of 0.5 µg / ml and mixed well. The 25 µl of the amplified products were mixed with 2 µl of 6X gel loading dye (0.25% bromophenol blue in 30% glycerol) and loaded in the slots of the submerged gel carefully with a micropipette. The last lane was loaded with 0.1 µg DNA of a molecular weight marker (100 bp) for determining the molecular weight of the amplified pro- ducts.

Gel documentation and analysis: The gels were observed under a UV light source in a gel documentation system (Alpha Imager, Alpha Innotech Corporation, San Leandro, CA), and the images of RAPD band patterns were stored in a computer for further analysis and future use.

The RAPD bands were scored as present (1) or absent (0) each of which was treated as independent character regardless of its intensity. The data was entered into binary matrix. Jaccard's coefficients of similarity (Jaccard, 1908) were calculated. The pair-wise similarity coefficients were calculated for all the genotypes using SIMQUAL module of NTSYSpc. All the analyses were done by using the computer package NTSYS-PC-2.02e (Rohlf, 1997) [12].

The dendrogram based on the similarity coefficients was obtained through the Un-weighted Pair Group Method with arithmetic Mean (UPGMA) (Sneath and Sokal, 1973) [15] and SHAN clustering. All the analyses were done by using the computer package NTSYS-PC-2.02e (Rohlf, 1997) [12].

Result and Discussion

The results pertaining to DNA finger printing of mungbean cultivars using RAPD markers are presented in Tables (Table 3 and Table 4) and Figures (Fig. 1 to 5).

The representative gel pictures of the electrophoretic pattern of PCR amplified DNA fragments of twelve cultivars of mungbean using RAPD primers OPA-02, OPA-11, OPN-10 and OPN-15 were shown in Fig. 1, Fig. 2, Fig. 3 and Fig. 4, respectively. A total 152 RAPD amplicons were obtained, out of which 108 were polymorphics (Table 3). Thus the average number of bands amplified per primer was 13.8. Lakhanpaul *et al*. (2000) [8], Lavanya *et al.* (2008) [9], Saini *et al*. (2010) [13] and Sony *et al*. (2012) reported 12.71, 24.85, 13.7 and 37.9 bands per primer in mungbean. Percentage polymorphism ranged from 23.08% (OPA 09) to a maximum of 100% (OPN 05 and OPN 16) across 11 primers with an average polymorphism of 71.05%. Six primers showed more than 85% polymorphism. The total number of polymorphic bands amplified was 108 (71.05%), with an average of 9.8 polymorphic fragments per primer. Datta *et al.* (2012) [2] reported the percentage of polymorphism ranged from 33% (OPX 5) to a maximum of 100% (OPX 4, OPX 6, OPX 13, OPX 15, OPX 19, OPD 5, OPD 7, OPD 20, OPI 4, OPI 6, OPI 13, OPI 14, OPI 18 and OPF 1), with an average polymorphism of 90% in 24 Indian mungbean cultivars using 33 RAPD markers. They also recorded an average of 7.0 polymorphic fragments amplified per primer.

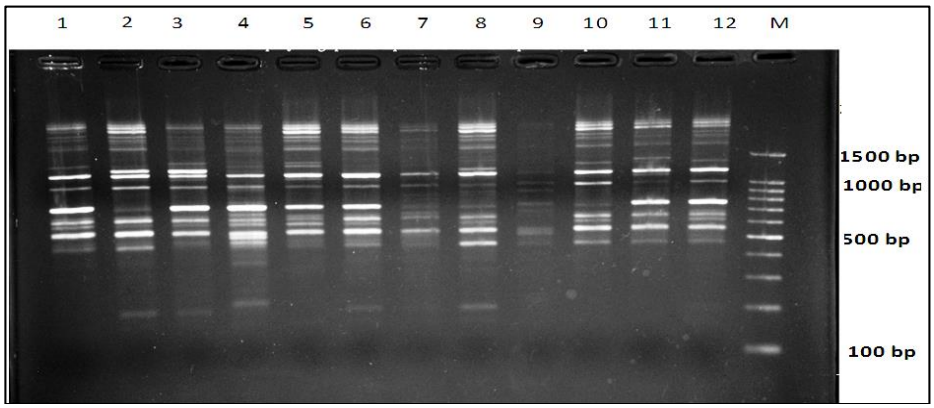

Fig 1: RAPD Profile of 12 Mungbean Cultivars using RAPD Marker OPA-02. M: Marker DNA, 100 bp Ladder. 1: Samrat, 2: BPMR-145, 3: HUM-2, 4: MUM-2, 5: Pairy Mung, 6: Pant M-2, 7: Pant M-4, 8: PKV AKM-4, 9: Pusa-0672, 10: Pusa - 9072, 11: RMG-62, 12: RMG-268.

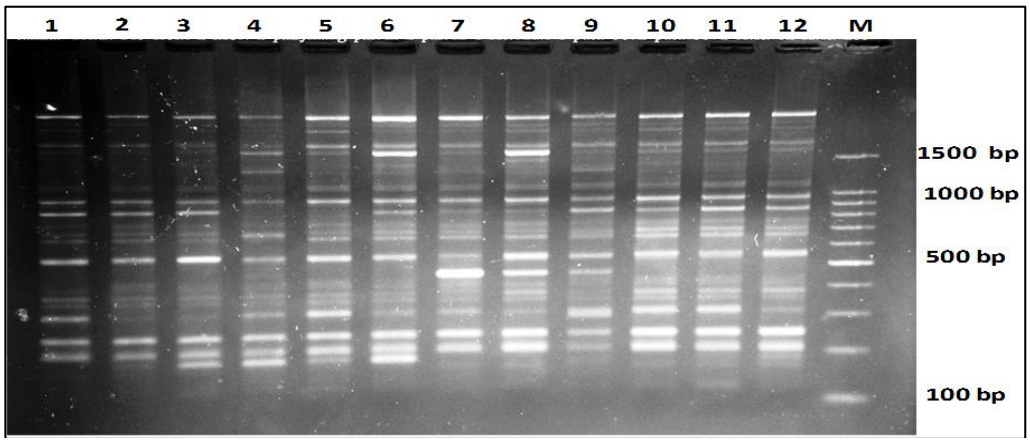

Fig 2: RAPD Profile of 12 Mungbean Cultivars using RAPD Marker OPA-11. M: Marker DNA, 100 bp Ladder. 1: Samrat, 2: BPMR-145, 3: HUM-2, 4: MUM-2, 5: Pairy Mung, 6: Pant M-2, 7: Pant M-4, 8: PKV AKM-4, 9: Pusa-0672, 10: Pusa - 9072, 11: RMG-62, 12: RMG-268.

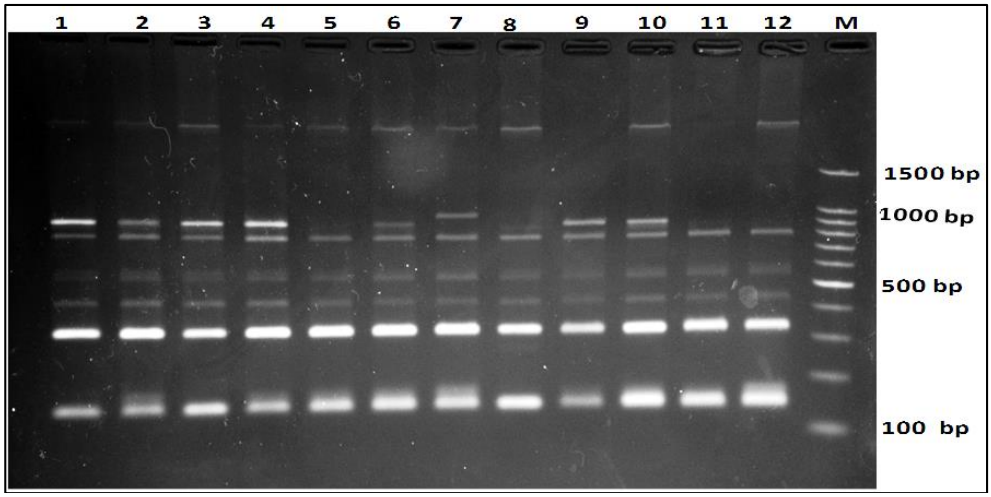

Fig 3: RAPD Profile of 12 Mungbean Cultivars using RAPD Marker OPN-10. M: Marker DNA, 100 bp Ladder. 1: Samrat, 2: BPMR-145, 3: HUM-2, 4: MUM-2, 5: Pairy Mung, 6: Pant M-2, 7: Pant M-4, 8: PKV AKM-4, 9: Pusa-0672, 10: Pusa -9072, 11: RMG-62, 12: RMG-268.

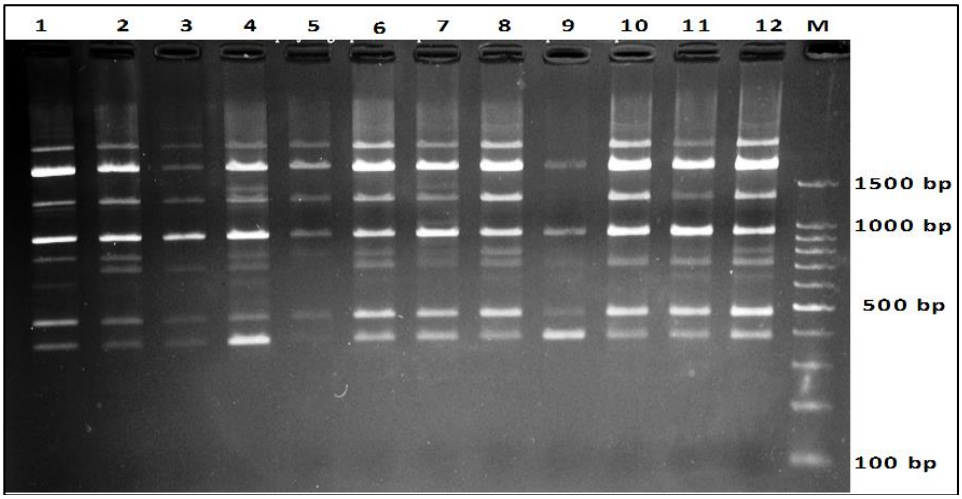

Fig 4: RAPD Profile of 12 Mungbean Cultivars using RAPD Marker OPN-15. M: Marker DNA, 100 bp Ladder. 1: Samrat, 2: BPMR-145, 3: HUM-2, 4: MUM-2, 5: Pairy Mung, 6: Pant M-2, 7: Pant M-4, 8: PKV AKM-4, 9: Pusa-0672, 10: Pusa - 9072, 11: RMG-62, 12: RMG-268.

Karuppanapandian *et al.* (2006) [7] analyzed genetic diversity in mungbean cultivars collected from various localities of Southern Tamil Nadu, India, using 20 RAPD decamer markers and reported average number of bands amplified per primer to be 10 with 83.0% polymorphism in 15 mungbean cultivars. Moreover, 12 unique bands were identified in different primer combinations. Among the responding primers OPA-11 generated maximum (22) number of bands with 36.36% polymorphism while primer OPA-14 as well as OPN-10 generated the minimum (8) number of bands with 87.50% and 37.50% polymorphism, respectively. Among the primers used, OPN-05 generated the maximum (20) number of polymorphic bands as well as highest (3) number of unique bands (Table3).

Table 3: Amplification Profiles of the RAPD Markers on 12 Mungbean Cultivars

Primer	Total number of bands	Polymorphic bands	Monomorphic bands	Unique bands	% of Polymorphic bands
OPA-02	15	13	2	2	86.67
OPA-09	13	3	10	1	23.08
OPA-11	22	8	14	0	36.36
OPA-14	8	7	1	0	87.50
OPN-05	20	20	0	3	100.00
OPN-08	12	5	7	1	41.67
OPN-10	8	3	5	1	37.50
OPN-13	15	14	1	0	93.33
OPN-14	10	9	1	1	90.00
OPN-15	13	10	3	2	76.92
OPN-16	16	16	0	1	100.00
	152	108	44	12	71.05

Jaccard's similarity coefficients (Table 4) were calculated to assess the genetic resemblances among the cultivars and the similarity coefficients matrix was used for UPGMA cluster analysis. The pair wise genetic similarity coefficients among 12 mungbean cultivars varied from 0.60 (Pusa 0672 versus MUM-2) to 0.94 (RMG-268 versus RMG-62). The RMG-62 and RMG-268 found to have maximum similarity, followed by RMG-268 and Pant M-2. The cultivar Pusa-0672 and MUM-2 showed maximum variability.

Table 4: Jaccard's Similarity Coefficient Values among 12 Mungbean Cultivars based on RAPD Data

Cultivars	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-11	C-12
C-1	1.00											
C-2	0.82	1.00										
C-3	0.77	0.86	1.00									
C-4	0.81	0.77	0.79	1.00								
C-5	0.78	0.83	0.82	0.79	1.00							
C-6	0.86	0.84	0.82	0.85	0.80	1.00						
C-7	0.76	0.79	0.73	0.76	0.77	0.83	1.00					
C-8	0.81	0.83	0.77	0.78	0.77	0.87	0.86	1.00				
C-9	0.65	0.66	0.63	0.60	0.66	0.70	0.63	0.69	1.00			
C-10	0.86	0.80	0.81	0.81	0.80	0.86	0.83	0.85	0.64	1.00		
C-11	0.79	0.80	0.79	0.79	0.82	0.85	0.84	0.85	0.67	0.84	1.00	
C-12	0.82	0.85	0.80	0.82	0.88	0.89	0.84	0.86	0.69	0.85	0.94	1.00

C1: Samrat, C2: BPMR-145, C3: HUM-2, C4: MUM-2, C5: Pairy Mung, C6: Pant M-2, C7: Pant M-4, C8: PKV AKM-4, C9: Pusa-0672, C10: Pusa-9072, C11: RMG-62, C12: RMG-268.

The dendrogram (Fig. 5) revealed the genetic similarity among the twelve cultivars of mungbean which ranged from 0.66 to 0.94, but mostly concentrated between 0.80and 0.88. This indicated a rather narrow genetic base of tested mungbean cultivars. The RAPD cluster pattern is presented in Fig. 5 segregated the 12 mungbean cultivars into two major clusters. The cultivar Pusa-0672 formed Cluster 1 (C-1) and cultivars Samrat, BPMR-145, HUM-2, MUM-2, Pairy Mung, Pant M-2, Pant M-4, PKV AKM-4, Pusa-9072, RMG-62 and RMG-268 have made Cluster 2.

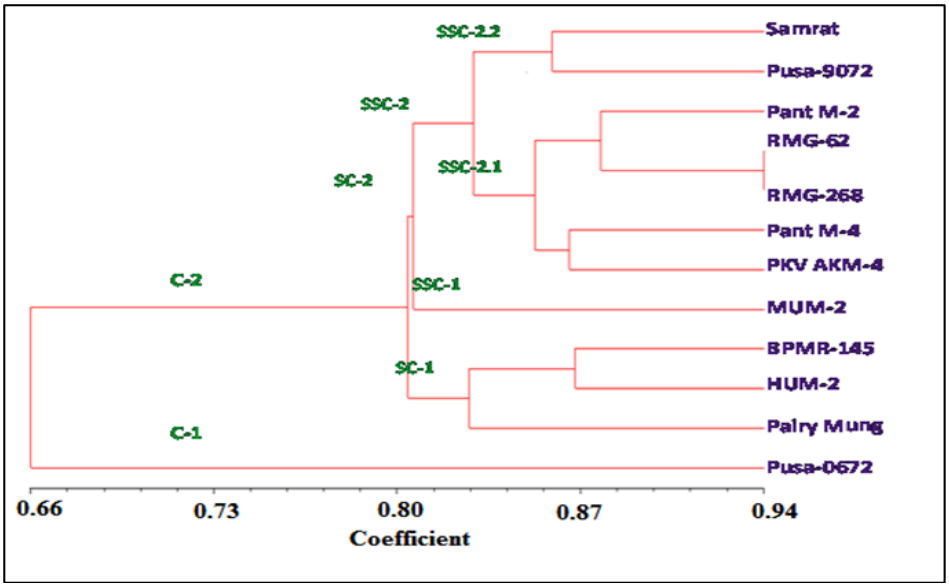

Fig 5: Dendrogram derived from UPGMA Cluster Analysis showing Genetic Relationship among 12 Mungbean Cultivars based on Random Amplified Polymorphic DNA

In Cluster 1, Cultivar Pusa-0672 formed individual group with distinctly separated from the cultivars of Cluster 2. The Cluster 2 formed two Sub Clusters (SC-1 and SC-2). The SC-1 comprised of two groups. The first group comprised of BPMR-145 and HUM-2; while Pairy Mung formed second group distinctly separated from the first group. The SC -2 was further divided into two- Sub Sub Cluster-1 (SSC-1) and Sub Sub Cluster-2 (SSC-2). The cultivar MUM-2 formed SSC-1, which was distinctly separated from remaining seven cultivars (Samrat, Pusa-9072, Pant M-2, RMG-62, RMG-268, Pant M-4 and PKV AKM-4) of SSC -2. The SSC-2 was further fractioned into two- SSC 2.1 and SSC 2.2. The SSC 2.1 comprised of 3 groups. The cultivar Pant M-2 individually formed first group which was separated from remaining two groups. The second group was comprised of RMG-62 and RMG-268, which were not separated from each other, while the third group comprised of Pant M-4 and PKV AKM-4, they are very close to each other. The SSC 2.2 comprised of only one group, where Samrat and Pusa-9072, are present and are very much close to each other. It is evident from pedigree that RMG-62 and RMG-268 have one parent (R-288) in common, and they were clustered in same group in SSC 2.1, moreover they developed from same geographical location (RAU, Durgapura).

All the 108 polymorphic fragments scored were used for genetic diversity analysis. Jaccard's similarity coefficients were calculated to assess the genetic resemblances among the cultivars and the similarity coefficient matrix was used for UPGMA cluster analysis. The cluster analysis separated 12 cultivars into two distinct groups. Clustering of mungbean cultivars into two groups showed reasonable variability that may be exploited for selecting parents for breeding purposes.

The use of appropriate statistical 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. This method has been employed in present study during analysis of RAPD polymorphism and thus the clusters obtained are reproducible.

The foregoing discussion clearly indicates that each of the 12 mungbean cultivars possessed specific RAPD finger printing profile. Thus, RAPD analysis proved to be applicable for genotyping, genetic diversity and relatedness evaluation. The analysis revealed narrow genetic base among cultivars used in this study. The narrow genetic base of cultivars of mungbean observed in this study is in conformity with other reports (Santalla *et.al.* 1998 and Lakhanpaul *et al*., 2000) [14, 8], emphasizing the need to broaden genetic variation by utilization of diverse germplasm collections. Wild relatives, exotics and mutant lines appeared to be good sources for genetic variation. Pre breeding or genetic enhancement needs emphasis for the transfer or introgression of genes and gene combinations from unadapted sources into more useable breeding material.

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