RESEARCH NOTE

Molecular and morphological characterization of fixed lines from diverse cross in mung bean (*Vigna radiata* (L.) Wilczek)

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Introduction

Mung bean is an important crop grown in 2.99 million hectare with 1.02 million tonne production (average during 2000–2003). This crop provides protein-rich food, restores and maintains the soil fertility by fixing atmospheric nitrogen, and also fits well in different cropping systems. However, the productivity of mung bean is only around 350 kg per ha. The low productivity of this crop can be attributed to narrow genetic base (resulting in low yield potential and susceptibility to biotic and abiotic stresses) and lack of suitable plant types for different cropping situations. Mung bean is suitable crop for diversifying more than 10.5 ha land under wheat–rice cropping system (Ali 2004) in 'Indo-Gangetic plain' and for sustaining the present productivity levels of cereals. The desired maturity duration for spring/summer season is 58–62 days, with determinate growth habit, high harvest index, photo-insensitivity and thermo-insensitivity, high pod set in first reproductive flush along with concentration at the top of the plant of pods that remain nonshattering, fast initial growth, long pod with above 10 seeds per pod and 1000-seed weight of 35–40 g. Short duration mung bean varieties require determinate growth habit. The present investigation was conducted with the specific objective of identifying suitable parents from advanced lines of diverse cross for hybridization with established agronomic base PDM-139. For developing suitable varieties for cultivation in spring/summer season, both morphological and molecular analyses were carried out for characterization of advanced breeding lines.

Materials and methods

Fifteen advanced breeding lines of mung bean including mung bean yellow mosaic resistant and short duration variety PDM-139 were included in the present study. The advanced lines studied are derived from a cross between IPM-99-125 × Pusa bold-I. Pusa bold-I is bold seeded selection from NM-92 and is released as Pusa Vishal for cultivation in spring season in northwest-plain zone, whereas IPM-99-125 is a mung bean variety evolved from interspecific cross Pant mung-2 × AMP 36 (urd bean), and is recommended for cultivation in northeast-plain zone of country.

The morphological data were generated by evaluating the material at two locations: Indian Agricultural Research Institute, New Delhi, and Indian Institute of Pulses Research, Kanpur, in spring season during 2004. At both the locations, the experimental material was sown in two replications of four rows/entry, and row length being 4 m. The recommended practices were followed to raise the crop. Ten plants were randomly selected to record the observation on pods per plant, height of the lowest fruiting node, seeds per pod, pods per cluster, pod length, length of the fruiting zone, yield per plant, 100-seed weight and plant height. The observations on days to 50% flowering and maturity were recorded on plot basis. The D^2 analysis as suggested by Mahalanobis (1936) and Tocher's method (Rao 1952) was followed for clustering of genotypes based on morphological traits. The molecular analysis was carried out at the National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi.

DNA extraction

Young leaf tissue (5 g) from five-day-old seedlings was ground in liquid nitrogen by using pre-chilled mortar and pestle, and the DNA was isolated by cetyl trimethyl ammonium bromide (CTAB) method (Torres *et al*. 1993). Leaf tissue powder was transferred to a polypropylene tube containing 25 mL of DNA extraction buffer (50 mM Tris-HCl,

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150 mM NaCl, 100 mM EDTA, 10% CTAB and 2 μL of β-mercaptoethanol) and incubated at 65°C for 30 min with occasional gentle swirling. Two-third volume of chloroform : isoamyl alcohol (24:1, v/v) were added and mixed. The samples were stored at −20°C for 15–25 min and centrifuged at 10000 rpm for 10 min at 4°C in Rotor RC 5B Plus centrifuge (Thermo Scientific, Waltham, USA). DNA pellet was rinsed with 70% ethanol for 10–15 min and dried at room temperature overnight. The pellet was dissolved in TE buffer (pH 8.0) and subjected to RNAase treatment for purification. The quantification of DNA was performed by running 2 μL of each DNA sample on 0.8% agarose gel along with uncut lambda DNA (30 and 60 ng) adjust to final concentration of 20–25 ng in 2 μL for use in PCR analysis.

Polymerase chain reaction

Forty RAPD primers (from kits OPC, OPE, OPF and OPG), were obtained from Operon Technologies, Almeda, USA. Five universal rice primers (URPs) originally derived from the repeat elements of weedy rice by Kang *et al*. (2002) were synthesized from the Operon Technologies, Almeda, USA. One SSR primer designed from the ferritin gene (acc. no. X58274) was derived from *Phaseolus vulgaris*. Fourteen primers consisting of eight RAPDs, five URPs and one SSR were selected for the final analysis of advanced breeding lines as other primers produced sub-optimal and indistinct amplification products in the initial experiments on primer survey. The list of primers, their sequences and annealing temperature, along with some of the characteristics of the amplification products are given in table 1.

For molecular analysis, PCR amplifications were carried out in a total volume of 25 μL containing 25–30 ng of genomic DNA (Willams *et al*. 1990). The PCR reaction mixture consisted of 10× PCR buffer (0.01% gelatin, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 10 mM dNTPs, 0.2 U *Taq* DNA polymerase and 0.2 μM primer. All the PCR reaction components were obtained from Bangalore Genei, Bangalore, India.

The PCR amplification was performed by using Thermal cycler (T-Gradient Biometra, Göttingen, Germany). For RAPD analysis, PCR temperature profiles were used as initial DNA denaturation at 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min. Final cycle at 72°C for 7 min was also performed. For both URP and SSR markers, PCR reactions were performed with initial DNA denaturation at 94°C for 5 min followed by 35 cycles at 94°C (1 min) for DNA denaturation, 45°C and 42°C (1 min) for primer annealing for URP and SSR markers, respectively, 72°C (2 min) for primer extension and final extension at 72°C for 7 min was performed. All the amplified PCR products obtained from RAPD and URP markers were resolved by electrophoresis on 1.4% agarose gel and SSR marker at 3.5% agarose gel for 3 h in 1× TBE buffer, stained with ethidium bromide, and the photographs taken using Gel Documentation System (Alpha Innotech, San Leandro, USA).

Table 1. Primers, sequence, annealing temperature and total number of loci amplified, number of polymorphic loci and Rp of primer.

Primer	Sequence (3′ − 5′)	Annealing temperature	Total no. of loci amplified	No. of polymorphic loci	Rp
RAPD					
OPG13	CTCTCCGCCA	35°C	6	4	5.000
OPF13	GGCTGCAGAA	35°C	6	2	10.000
OPC4	CCGCATCTAC	35°C	8	6	9.125
OPC11	AAAGCTGCGG	35°C	8	5	6.625
OPC13	AAGCCTCGTC	35°C	7	4	6.750
OPC16	CACACTCCAG	35°C	7	4	6.250
OPE5	TCAGGGAGGT	35°C	6	3	10.975
OPE11	GGTGACTGTG	35°C	3	1	4.250
					Mean Rp = 7.371
URP					
6F	GGCAA GCT GGT GGG AGG TAC	45°C	6	4	6.250
2F	GTGT GC GAT CAG TTGC TGGG	45°C	6	4	6.375
2R	GCC AGC AACT GAT CGC AC AC	45°C	3	1	4.250
17R	AA TGT GGG CAA GC TGG TGGT	45°C	4	3	6.125
13R	TAC ATC GCA AGT GACA CACC	45°C	7	6	6.375
					Mean Rp = 5.875
SSR					
FER	TC GCA AA GTT GCCA GTC AGT TAG AAGG AAGG AGGGC ATG	42°C	5	3	6.125

Rp, resolving power.

Data analysis

DNA bands obtained from RAPD, URP and SSR markers were scored visually for the presence (1) and absence (0) of bands for all studied genotypes. The resolving power (Rp) of each primer was calculated as Rp = ΣIb, where Ib (band informativeness) takes the value of: $1[2\times(0.5-p)]$, p being the proportion of genotypes of different *Vigna* species containing that band (Prevost and Wilkinson 1999).

Genetic diversity between any two samples i and j was estimated by using the Dice similarity coefficient (GS_{ij}) = $2a/(2a+b+c)$, where a is the number of polymorphic bands shared between genotypes i and j and b is the number of bands present in i but absent in j, whereas c is the number of bands present in j but absent in i. The term $2a + b + c$ represents the total number of fragments analysed in all the samples using NTSYS-PC software (Rohlf 2000). Sequential agglomerative hierarchial nested (SAHN) clustering was performed on the similarity matrix using Dice coefficient utilizing the unweighted paired group method with arithmetic averages (UPGMA).

Results and discussion

Molecular analysis

DNA marker data obtained from check variety PDM-139 and PDM-15 advanced lines of cross IPM-99-125 × Pusa bold-2 (IPM-02-1 to IPM-02-15) of mung bean with eight RAPDs, five URPs and one SSR markers were analysed. A total of 51 loci were amplified, of which 29 were polymorphic with RAPD. The number of polymorphic loci ranged from one COPC 11 to six COPC 4. The Rp ranged from 10.925 for OPE-5 to 4.250 for OPE-11. The mean Rp of RAPD's were 7 : 371. In case of URP's, a total of 26 loci were amplified of which 18 were polymorphic. The number of polymorphic loci varied from one (URP 2R) to six (URP 13R) and Rp was from 6.375 (for primers URP 13R and URP 2F) to 4.250 (for primer URP 2R). The mean Rp of URP's was 5.875. SSR marker developed from the ferritin gene of *P. vulgaris* is a multilocus marker, which amplified five loci out of which three were polymorphic and the Rp of this primer was 6.125.

Fourteen DNA markers used for molecular analysis of 15 advanced lines of diverse cross in mung bean and PDM-139 check variety exhibited polymorphism in studied genotypes. The combined marker data set obtained from RAPD, URP and SSR markers over 82 loci were analysed for pairwise genetic similarities based on UPGMA method, DNA finger print profiles of PDM-139 (control) and 15 genotypes of *V. radiata* obtained with RAPD primer OPG-13 (figure 1). On the basis of the combined data set, five clusters were obtained for 16 studied genotypes. Cluster I comprised a single genotype, IPM-02-1. Cluster II had maximum 12 genotypes with 89% genetic similarity. Clusters I, III and IV revealed one genotype each. The check variety PDM-139 was now grouped in cluster V. The maximum intercluster diversity was recorded between clusters V and II. The variety PDM-139 used as standard check was grouped separately. The genetic variations among advanced lines of diverse cross could be useful for selecting parents for crossing for generating appropriate populations required for breeding for yield and related agronomic traits.

On the basis of evaluation of morphological data, the studied lines were grouped into five clusters (see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). Cluster I comprised of IPM-02-5, 13, 11, 3 and 8 and cluster III also comprised of five genotypes viz., IPM-02-4, 10, 2, 12 and 15. Cluster II (IPM-02-1, 9 and 7) and cluster V (IPM-02-6 and 14) comprised of three and two genotypes, respectively. Check variety PDM-139 was grouped separately in cluster IV. Cluster V exhibited maximum intracluster distance. The genotypes of this cluster were diverse. The selection pressure applied during segregating generation led to differentiation. Cluster IV exhibited minimum intercluster distance as there was only one genotype in this cluster. The clusters exhibiting maximum intercluster distance were II and V, followed by I and V and IV, and V. IPM-02-6 and IPM-02-14 could be crossed with genotypes of cluster IV, II and I for maximum variability

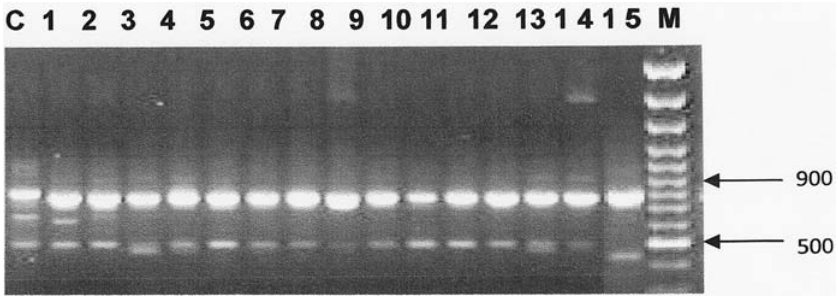

Figure 1. DNA finger print profiles of PDM-139 (control) and 15 genotypes of *Vigna radiata* obtained with RAPD primer OPG 13 along with 100 bp plus DNA ladder (M).

Table 2. Average intercluster and intracluster distances.

	Cluster I	Cluster II	Cluster III	Cluster IV	Cluster V
Cluster I	6.43	8.99	11.99	10.93	21.35
Cluster II		6.06	11.97	10.50	25.35
Cluster III			7.49	11.57	17.78
Cluster IV				0.00	21.14
Cluster V					8.0

for the effective selection. The cluster mean for studied traits are shown in table 2 of electronic supplementary material. The cluster exhibiting maximum intercluster distance were II and V, followed by I, V and IV, V (table 2). None of the clusters contained any genotype with all the desirable character which could be directly selected and utilized as a commercial variety. To obtain desirable plant types, recombination among genotypes of diverse clusters is necessary. The best cross combinations based on morphological and molecular data analyses are PDM-139 × IPM-02-6 and PDM-13 × IPM-02-14. These cross combinations are likely to release segregants suitable for short duration cultivation in spring/summer season.

Limited studies have reported on the use of molecular markers for diversity analysis in *Vigna* species (Santalla *et al*. 1998; Lakhanpaul *et al*. 2000; Lambridges and Lawn 2000; Ranade and Gopalkrishna 2001; Souframanien and Gopalkrishna 2004). Santalla *et al*. (1998) used RAPD markers for the analysis of genetic diversity in 19 landraces of cultivated mung bean (*V. radiata* L. Wilczek) and weedy and wild relatives including *V. luteola* and *V. radiata* var. *sublobata* and urd bean (*V. mungo*). Lakhanpaul *et al*. 2000 used 20 RAPD markers to analyse diversity in 32 Indian mung bean cultivars. The clusters revealing greater homology were obtained. Ranade and Gopalkrishna (2001) carried out diversity analysis in 20 urd bean cultivars using RAPD markers. Souframanien and Gopalkrishna (2004) used 25 RAPD and 16 ISSR primers to study 18 genotypes of black gram and found that ISSR markers were more efficient in comparison with RAPD primers. Lack of PCR based markers like sequence tagged microsatellite markers site (STMS) and simple sequence repeats (SSR) are the main bottlenecks for extensive molecular analysis in mung bean.

In present study, attempt was made to combine RAPD, URD and SSR markers derived from other plant species for diversity analysis in selected advanced lines of diverse cross in mung bean. The results based on morphological data generated at two locations IARI, New Delhi and IIPR, Kanpur, were correlated with molecular data. Based on these data sets, the crosses were suggested identifying suitable prebreeding lines specifically for evolving the breeding material for identifying suitable lines for mung bean cultivation in spring summer season.

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