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Genetic Diversity Analyses of Mungbean (*Vigna radiata* [L]. Wilczek) by ISSR

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ABSTRACT

In the present study 3′ anchored ISSR primers were designed, and after optimizing each primer annealing temperature for obtaining a higher number of markers per primer, they were used to analyze genetic diversity among 87 mungbean genotypes from India and neighboring countries. Following screening the 24 ISSR primers, 8 primers that yielded a clear banding pattern were chosen. Out of a total of 145 bands, 135 were polymorphic. An average of 18.12 bands and 16.87 polymorphic bands per primer was obtained. A dendogram revealed clustering of genotypes into 5 clusters. The first two clusters individually included the genotypes developed at two of the agricultural research institutes in India. Cluster three included genotypes developed in and around India, and cluster 4 included the genotypes that were local collections of mungbean from India and the fifth cluster included exotic and old selections of mungbean. Analyses showed that both resolving power of primer and number of polymorphic bands showed a positive correlation with the number of genotypes identified.

Keywords: exotic varieties, genetic diversity, PCR optimization
Abbreviations: ISSR, inter simple sequence repeat; **Rp**, resolving power; **PCR**, polymerase chain reaction; **UPGMA**, unweighted pair group method with arithmetic average

INTRODUCTION

Mungbean (*Vigna radiata* [L]. Wilczek), also known as green gram or moong, is grown in the Asian subcontinent. In India, it is one of the major pulse crops grown on about 3 million ha in arid, semi-arid and sub-humid regions. Mungbean is native to India (Paroda and Thomas 1987). It is the third important legume crop in India after chickpea and pigeon pea. In India, mungbean is cultivated on 3.77 million ha and production is 1.56 million tonnes with an average yield of 413 Kg/ha during 2007-2008 (Singh 2009). The productivity varies year to year and among states (Singh 2009). In general, there is scope for the improvement of yield as productivity up to 800 tonnes/ha is reported from the state of Punjab (Singh 2009). Although the germplasm collections from India are very large, much diversity has not been reported on the basis of morphological characters. There is an urgent need to identify genetic diversity based on a molecular basis for utilization in breeding programmes.

Conventional morphological characters, isozyme and other biochemical markers have been used to examine genetic diversity in mungbean (Mohanty *et al*. 2001). But most of the breeding programs would require markers that can be conveniently used for tagging multiple genes for a phenotype, for identification of a variety, for genetic mapping studies and to determine the genetic diversity in the germplasm collection (Kumar 1999). In the recent past DNA-based molecular markers have been applied for various purposes including assessment of genetic diversity, mapping of the genome and studying the species relationship within the genus *Vigna*. Genetic diversity among mungbean genotypes has been reported using different DNA markers (Lakhanpaul *et al*. 2000; Kumar *et al*. 2003; Afzal *et al.* 2004; Saini *et al*. 2004; Bhat *et al*. 2005; Seehalak *et al*. 2006; Sangiri *et al*. 2007; Reddy *et al.* 2008; Chattopadhyay *et al*. 2008; Lavanya *et al*. 2008; Saini *et al*. 2008; Dikshit *et al*. 2009; Saini *et al.* 2010; Ullah *et al.* 2010; Tantasawat *et al*. 2010; Vir *et al.* 2010; Narasimhan *et al.* 2010; Raturi *et al*. 2011; Taunk *et al*. 2012). RAPD analysis has been used to study relationship among the species belonging to *Ceratotropis* (Kaga *et al*. 1996). Other marker such as AFLP was used for genetic diversity analyses of mungbean (Bhat *et al*. 2005) and other species of *Vigna* (Tomooka *et al.* 2002). The presence of two types of internal transcribed spacer (ITS) among mungbean genotypes based on the methylation status of a *Bam*HI site present in the ITS region, were reported by Saini *et al*. (2000) and further analyses indicated the presence of multiple intragenomic ITS variants showing a high genetic diversity in mungbean (Saini *et al*. 2008). Recently the development of sequence tagged microsatellite sites and their use in analyzing a set of 87 genotypes has been reported (Singh and Jawali 2009), which included 48 genotypes that were used in genetic diversity analyses by AP-PCR (Saini *et al*. 2004).

Among a large number of PCR-based methods available for assessing genetic diversity in plants (Kumar 1999), ISSR, which is based on microsatellite sequences and does not need flanking sequence information, is easy to develop. ISSRs are highly reproducible compared to RAPD. This reproducibility is attributed to the length of the primers and higher annealing temperature used in the ISSR analysis. ISSR has been used extensively in studying a variety of aspects of plant biology (Rakoczy-Trojanowskam and Bolibok 2004).

The objective of the present study was to (a) optimize annealing temperature for each primer in PCR to achieve a larger number of markers per primer, (b) to use these for assessing the genetic diversity among the 87 mungbean

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genotypes that were used in our previous study (Singh and Jawali 2009) and (c) formulating a set of primers for the purpose of identifying the genotypes in this collection.

MATERIALS AND METHODS

The mungbean genotypes used in the present study are listed in **Table 1**. The DNA was isolated from seeds imbibed for 24 h according to Krishna and Jawali (1997). The DNA preparation was treated with RNAse, further purified and quantified as detailed by Prasad *et al*. (1999).

Design of primers

An ISSR primer with inclusion of only one base extension at the 3′ end when used for PCR, results in a large number of bands which often appears as a smear on an agarose gel. However, anchoring base(s) at either 3′ or 5′ end of the primers reduces the number of products (Gupta and Varshney 2000). Hence, in the present study, 25 ISSR primers (18-20 base long) with two nucleotide anchors and 2 primers (21 bases long) with three nucleotide anchors at the 3′ end were used (**Appendix 1**). Generally, the dinucleotide repeat motifs are present in reasonable abundance, hence, the majority of the primers designed were based on these repeats. The other primers chosen arbitrarily included two trinucleotide repeats and five tetranucleotide repeats.

PCR amplification

The reactions were performed in 25 µl in an Amplitron II Thermolyne cycler (Barnstead/Thermolyne, Dubuque, IA, USA). The reaction mixture consisted of genomic DNA: 50-100 ng; Tris-HCl (pH 9.0): 10 mM; $MgCl_2$: 2.0 mM; KCl: 50 mM; Gelatin: 0.01%; dNTP: 200 µM; primer: 0.5 µM and *Taq* DNA polymerase: 2U (Bangalore Genei Pvt. Ltd, Bangalore, India). After an initial denaturation step of 95°C for 5 min, the samples were incubated for 30 cycles of 95°C for 1 min, 40-60°C (depending on the primer used) for 1 min, 73°C for 1 min. and after a final extension at 73°C for 10 min and was stored at 4°C until use. Amplification products were separated on a 2% agarose gel (10 cm × 15 cm) using 1X TBE at a constant voltage of 100 V. The DNA fragments were visualized by staining with ethidium bromide (Singh and Jawali 2009) and photographed.

PCR was carried out at 4 or 5 annealing temperatures for each of the primers to identify the condition for obtaining maximum polymorphic DNA bands. Since the primary aim was to obtain higher number of markers per primer, the effect of annealing temperature on the PCR product profile and on the polymorphic band was analyzed on six diverse genotypes (TARM-18, RUM-5, JL-781, KOPERGOAN, PDM-116 and ML-5).

Reproducibility of ISSR profile

The reproducibility of the bands was ascertained by carrying out analyses at least twice and also across the experiments that included genotype-ISSR primer combination for different PCR runs. Further, in a second experiment, DNA was isolated from at least 15 genotypes *viz*: TARM 18, TARM 2, RUM5, JL-781, Kopergoan, PDM-1, PDM-54, WGG-13, LGG-450, PDM-116, ML-5, E-11-18, PUSA-22, PUSA-104, PUSA-105, and used for ISSR analyses. Results showed that under the conditions employed the polymorphic bands were reproducible. Similarly, DNA isolated independently four times for two (TARM-2 and Kopergoan) of the genotypes yielded profiles that were highly reproducible.

Data analysis

For each ISSR primer, PCR amplification was repeated at least twice and all the bands that were polymorphic in both runs were recorded. For at least 15 accessions, the DNA was isolated a second time and the ISSR analyses were carried out as indicated above. Each band obtained was considered as a separate marker and scored as either present (1) or absent (0) across all genotypes (Gherardi *et al*. 1998). The size of the DNA fragment was estimated using the relative migration distance of a DNA ladder.

Genetic relatedness and genotype identification

The binary data of the marker–genotype matrix was used for cluster analysis of the genotypes using TREECON version 1.3b (Van der Sande *et al*. 1992; Van de Peer and Wachter 1994). The Nei and Lei similarity coefficients, estimates of genetic identity, between varieties were evaluated, using a pair-wise comparison based on the proportion of bands shared by the genotypes (Nei and Lei 1979).

$$\text{Similarity coefficients (S)} = 2N_{ab} / (N_a + N_b)$$

where N_a and N_b represent total number of bands present in genotypes A and B under consideration, and N_{ab} is the number of bands shared by both the genotypes. The similarity matrix was utilized to construct an UPGMA dendrogram.

The identification of a genotype was achieved by analyzing the band profiles obtained by using a given primer or a set of primers to have at least one different amplified DNA fragment among the two genotypes under consideration.

The resolving power (R_p) of a primer was calculated as the sum of band informativeness (I_b). All the polymorphic bands of a primer was scored as described by Prevost and Wilkinson (1999) using the equation

$$R_p = \Sigma I_b$$

I_b is represented in a 0-1 scale is obtained by the formula:

$$I_b = 1 - (2 \times |0.5 - p|),$$

where "p" is the proportion of the 87 genotypes containing the band. The I_b value was calculated for all 68 polymorphic ISSR bands scored in the study from which the R_p for the individual primers was calculated.

RESULTS

Primer screening and optimization of PCR conditions

In the first step, out of 27 primers analyzed (**Appendix 1**) for the ability to produce PCR amplification products with TARM-18 at the theoretical annealing temperature 24 gave successful amplification and of these eight primers (**Table 2**) yielded a good profile. In the second step, PCR conditions were further optimized to achieve a higher number of discrete and detectable bands as well as to obtain a higher number of polymorphic bands. Since the primary aim was to obtain a higher number of markers per primer, the effect of annealing temperature on the PCR product profile and on the polymorphic band was analyzed on six genotypes (TARM-18, RUM-5, JL-781, KOPERGOAN, PDM-116 and ML-5) and the results for two of the 7 primers are given in **Table 3**. The profiles obtained using two primers are shown in **Fig. 1** and **Fig. 2**. Based on the effect of temperature on the band profiles, the primers can be grouped into two categories. Primers in the first category represented by GGTT5 (**Fig. 1, Table 3**) which at low temperature (40°C) yield a large number of bands along with a smear in the background, making the analysis difficult. At a slightly higher temperature (45°C), the background smear was reduced with no significant difference in the detectable bands, thus making the analysis easier. The polymorphic bands with respect to numbers were not much different, however the number of polymorphic bands obtained did not vary much with the increase of annealing temperature; however, the DNA band profiles became discrete and detectable with less background smear. In the second category, represented by GA1 primer (**Fig. 2, Table 3**), total number of discrete bands remained the same at both 45 and 50°C but the number of polymorphic bands was higher at 50°C. However, with a further increase of annealing temperature to 55 and 60°C, there was an increase in both the number of bands and polymorphic bands. **Table 2** shows the details of the

Table 1 Mungbean genotypes used in the study.

Mungbean genotypes	Pedigree/source	Characteristics
TARM-1	RUM 5 X TPM1 Trombay, BARC, India	Late flowering and powdery mildew resistance
TARM-2		
TARM-13		
TARM-18	PDM54 X TARM2 Trombay, BARC, India	
TARM-21	RUM 5 X TPM1 Trombay, BARC, India	
TARM-22		
TARM-26		
TARM-32		
TARM-35		
TPM-1		
E-11-18	PDM 54 X TARM2 Trombay, BARC, India	Early maturing
E-11-24		Powdery mildew resistant
E-24-26		
E-26		
E-28		
E-29		
E-30		
E-31-5		
E-48		
E-65		
E-92-3		
PUSA-22	IARI, Delhi, India	
PUSA-SR-22		
PUSA-71		
PUSA-102		
PUSA-103	S-8 mutant X Cj-4 IARI, Delhi, India	
PUSA-104	Ps-16 X V3476 IARI, Delhi, India	
PUSA-105	Tainan X ML6 X EG-MG-16 X ML3 1 IARI, Delhi, India	Tolerance to YMV, powdery mildew, Macrophomia blight, aphids, white flies
PUSA-116	IARI, Delhi, India	
PUSA-117		
PUSA-168		
PUSA-90-11		
PUSA-90-31		
PUSA-90-71		
PUSA-91-31		
PUSA-93-71		
PUSA-93-72		
PUSA-94-72		
PUSA-95-31	Selection from NM-9473 IARI, Delhi, India	YMV resistant
PUSA-95-71		
PUSA-96-31		
PUSA-96-32		
PUSA-96-72		
PDM1	Selection of germplasm collected from Kundawa IIPR, Kanpur, India	Early maturing, YMV resistant
PDM-54		
PDM-84-131		
PDM-116		
ML-3	PAU, Ludhiana, India	Tolerant to powdery mildew
ML-5	No; 54 X Hyb 45 PAU, Ludhiana, India	Tolerant to YMV
Ml-6	PAU, Ludhiana, India	Early maturing
ML-127		
ML-337	Ml1 X ML 987 PAU, Ludhiana, India	
CO-5	KM2 X Mg-50-10 TNAU, Coimbatore, India	Tolerant to YMV
WGG-13	Warangal, Andhra Pradesh, India	
WGG-35		
WGG-48		
WGG-320		
LGG-410	LAM. Andhra Pradesh, India	
LGG-444		
LGG-450		
LGG-458		
MMG-316		
UPM-92-3		
NARP-1		
PIMS-1		
NN-19-19	Pakistan	Early maturing mutants and resistant to YMV
NN-94-73		
NN-20-21		
AKM-30	Maharastra, India	
NAYAGARH	Local variety, India	
GHAGA-1	Sriganganagar, India	
CHANTANPUR	-	

Table 1 (Cont.)

Mungbean genotypes	Pedigree/source	Characteristics
EC 337104	AVRDC, Bangladesh	
EC 318991		
PHULE M-1	Maharastra, India	
VC-6144-B-10	AVRDC, Bangladesh	Bold seed and tolerant to YMV
VC-6173-B-6		
VC-6173-B-13		
VC-3960-A-88		
VC-6173-C		
PUSABHARTI-IVTRII7	IARI, Delhi	
RUM-5	Raipur uteramung from Raipur, India	Powdery mildew resistant
JL-781	Selection from China moog	
KOPERGOAN	Local selection from Maharastra, India	Bold seed and high yielding variety
TAP-7	Mutant of S-8, BARC/Akola, India	Tolerant to powdery mildew
TM-97-25	Kopergoan X TARM 2 Trombay, BARC, India	

Table 2 Characteristics of band profiles obtained by the eight ISSR primers among 87 genotypes.

Primer	Primer sequence	Annealing temperatures (°C)	Total bands	Size range (bases)	Total polymorphic bands	No. of genotypes identified	Resolving power (Rp)
GA1	(GA)9(A/T)G	55	22	125-1400	22	63	8.02
GA5	(GA)9(G/T)G	50	15	200-1300	14	52	6.667
CA1	(CA)9(C/T)G	50	21	330-1300	21	39	4.85
CAA1	(CAA)6(G/T)A	40	16	180-1100	16	34	6.0
GAT1	(GAT)6(G/A)G	45	14	380-1250	11	21	4.94
GGTT2	(GGTT)4(G/A)T	40	17	290-2100	12	18	3.45
GGTT5	(GGTT)4(G/T)C	45	19	320-2200	19	52	6.48
GACA1	(GACA)4(C/T)G	40	21	200-2000	20	53	6.65
GAT1 and GGTT2					23	63	8.39
GAT1 and GA5					25	85	11.61
GAT1 and CAA1					27	72	10.94
GA5 and CAA1					30	83	12.7
GAT1 and GGTT5					30	82	11.42
GAT1 and GACA1					31	85	11.59
GAT1 and GA1					33	85	12.94
GA1 and CAA1					38	87	14.02

Table 3 Effect of annealing temperature on the ISSR profiles of six diverse mungbean genotypes.

Primer	Annealing temperature (°C)	Number of DNA bands	
		Total	Polymorphic
GGTT5	40	17	12
	45	16	13
	55	16	12
GA1	45	10	5
	50	10	7
	55	16	12
	60	14	11

optimized annealing temperature for the eight primers chosen for further analyses.

Characteristics of ISSR profiles

The eight ISSR primers yielded 14 to 22 detectable bands per primer and the size of the fragments ranged from 125 to 2000 bp (**Table 2**). The amplification profile of 23 out of 87 mungbean genotypes obtained using some primers is shown in **Fig. 3A-C**. Of a total of 145 bands, 135 were polymorphic among the 87 genotypes (**Table 2**). The number of polymorphic bands per primer varied from 11 to 22 with an average of 17. On average, the percentage of polymorphic bands exceeded 90%. Among these eight ISSR primers, CA1, CAA1, GA1, GA5, GGTT5 and GACA1 were highly polymorphic. While most of the other primers yielded more than 14 polymorphic bands, primer GA1 yielded the highest (22 bands). There was no correlation between the total number of bands produced and the number of polymorphic bands obtained. For instance, 100% polymorphic bands were obtained for four primers that produced a total of 16-22 bands all were found to be polymorphic while two other primers that produced 15 and 21 bands yielded more than 90% polymorphic bands, while another two primers that produced 14 and 17 bands yielded low percentage of bands being polymorphism (**Table 2, Fig. 3C**).

Estimation of genetic diversity

Using 135 polymorphic ISSR markers generated from the eight ISSR primers a rooted dendrogram based on UPGMA was obtained (**Fig. 4**). The 87 mungbean genotypes were grouped into 5 major groups. Group I included 20 of the 23 genotypes developed at BARC, Trombay. Group II included 20 of the 23 genotypes selected at PUSA, IARI from exotic mungbean, Group III consisted of mixed genotypes from India and neighboring countries. Group IV was comprised of the genotypes developed so as to fit into the cropping system in different parts of India. The last group consisted of old selections of mungbean that have not been used for production, but have been utilized in mungbean breeding programs.

Group I can be further divided into three subgroups based on their close relationship. Subgroup IA comprised of 10 genotypes (E series) obtained from a cross between PDM-54 (early maturing *kharif* crop) and TARM-2 (*rabi* crop having powdery mildew disease resistance genes and late maturing type) have powdery mildew resistance genes and other morphological characters similar to TARM-2 and early flowering ability alike PDM-54. Subgroup IB was comprised of six genotypes (TARM series) developed from a cross between RUM-5 and TPM-1, and most of these lines showed an inclination towards TPM-1 plant type with one or two morphological differences. These groups of lines

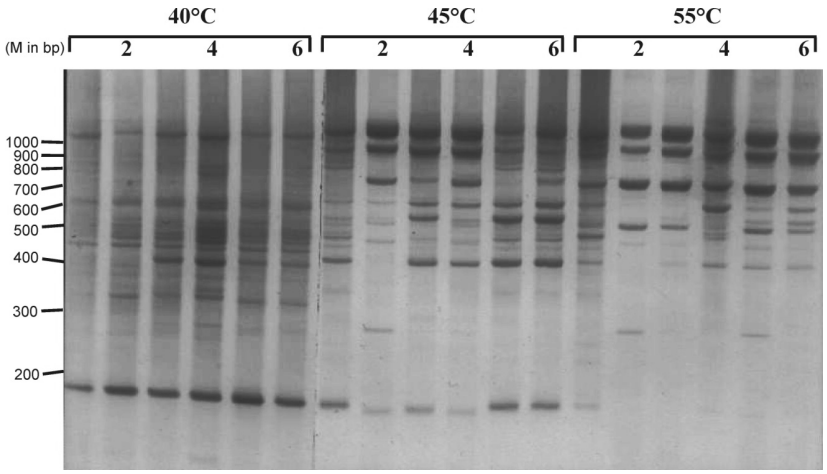

Fig. 1 Effect of annealing temperature on the ISSR profiles in six genotypes of mungbean. The DNA fragment profile from six genotypes of mungbean using GGTT5 primer at 40, 45 and 55°C. The lane 1 to 6 corresponds to mungbean genotypes viz. TARM-18, RUM-5, JL-781, KOPERGOAN, PDM-116 and ML-5, respectively. The molecular size of a DNA ladder is shown on the left of the figure.

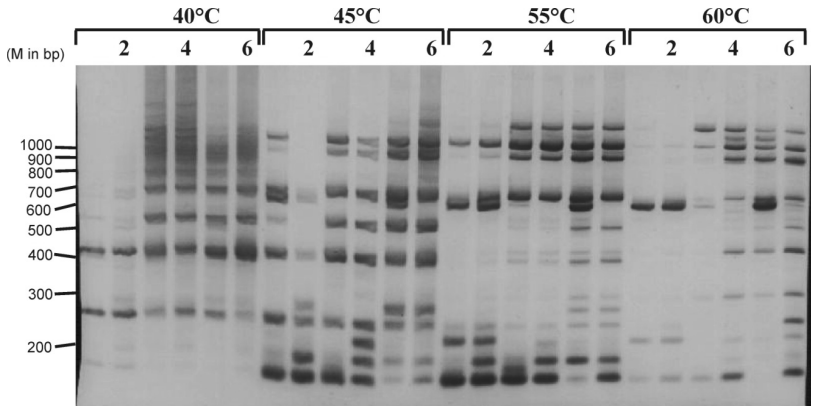

Fig. 2 Effect of annealing temperature on the ISSR profiles in six genotypes of mungbean. The DNA fragment profile from six genotypes of mungbean using in six genotypes of mungbean using GA1 primer at 45, 50, 55 and 60°C. The lane 1 to 6 corresponds to mungbean genotypes viz. TARM-18, RUM-5, JL-781, KOPERGOAN, PDM-116 and ML-5, respectively. The molecular size of a DNA ladder is shown on the left of the figure.

were suitable for the *rabi* season and were selected to have late flowering and powdery mildew resistance genes. As seen in the dendrogram, the TARM series, except for TARM-32 and TARM-35, belongs to one subgroup with TPM-1. Subgroup IC is comprised of 7 genotypes, some derived from a cross between RUM-5 and TPM-1. All these genotypes, including three PUSA genotypes, falling in this group are medium maturing genotypes.

Group II comprised of genotypes developed by either conventional breeding among the local genotypes or with exotic genotypes. Some of the genotypes are also derived from pure line selection. The pedigree of most of the lines is not available in the literature. However, TARM-18, which was obtained from a cross between PDM-54 and TARM-2, resembles PDM-54, shows a close relation with the PUSA genotypes.

Group III is comprised of 16 mixed genotypes of mungbean that were developed at national or international research institutes. For instance, Ghaga developed in Gujarat and AKM-30 and PHULE-M1 developed at Akola, Maharashtra represents the Indian mungbean genotypes. The five VC series developed at AVRDC, two EC from Taiwan and three NN series developed in Pakistan represents the international mungbean genotypes. TAP-7, UPM-92-3 and Chantanpur are exceptional because TAP-7 is derived from S-8 and the other two are local selections.

Group IV is comprised of 19 genotypes of mungbean, all of which are local collections of mungbean from India. All these genotypes have been introduced for cultivation under varying cropping seasons (cultivation in *kharif* (rainy season), *rabi* (winter season), spring, and summer) among the Indian states. For instance, the LGG and WGG series were suitable for *rabi* season in Andhra Pradesh while the ML series were suitable for *kharif* season in Punjab state. The *kharif* occupies larger area of cultivation and mostly depend on the rainfall and *rabi* is cultivated under irrigation or under residual moisture of the previous crop, where as spring and summer will be grown under irrigated condition. Each of the above cropping pattern, the temperature variation is very large.

The last group V includes only 5 genotypes of mungbean that are considered to be the old selections of Indian mungbean. These genotypes have not been altered over the years but at the same time have been used for genotype improvement by plant breeders. This group is comprised of RUM-5, TM-97-25, JL-781, KOPERGOAN and PUSABHARTI IVT-RII-7. Among them, RUM-5 and KOPERGOAN are old mungbean selections while JL-781 is a selection from China. TM-97-25 was obtained from the cross KOPERGOAN and TARM-2 and was selected for bold seed and other characters resembling KOPERGOAN. This genotype, along with PUSABHARTI IVT-RII-7 (whose pedigree is not known), was also clustered with the old mungbean selections. Genotype NAYAGARH, one of the old selections of Orissa, is placed the farthest of all 87 genotypes.

Genotype identification using ISSR markers

Considering at least one amplified DNA fragment to be different among the genotypes under consideration, genotypes have been identified by each ISSR primer. The number of genotypes identified by one ISSR primer varied from 18 to 63 (**Table 2**). GA1 primer identified the maximum number

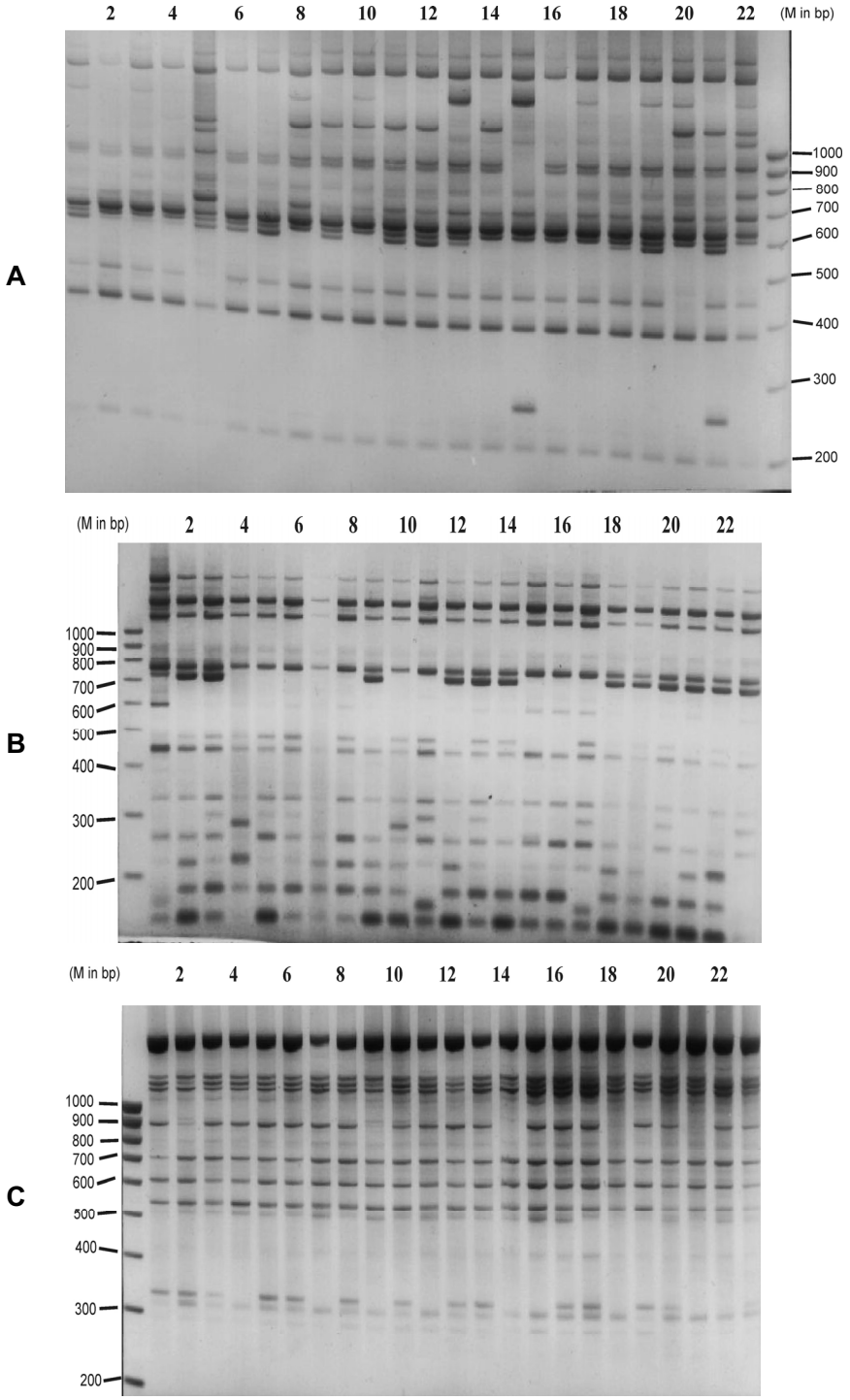

Fig. 3 ISSR banding profiles of mungbean genotypes using ISSR primer. The PCR products were separated on 2% agarose gel and lane M shows a DNA ladder in base pair (bp). (**A**) Profiles of mungbean genotypes obtained using GACA1 primer at an annealing temperature of 40°C. The lanes marked 1-22 corresponds to the genotypes numbered as 24 to 45 in **Table 1**. (**B**) Profiles of mungbean genotypes using GA1 primer at an annealing temperature of 55°C. The lanes marked 1-22 corresponds to the genotypes numbered as 47 to 68 in **Table 1**. (**C**) Profiles of mungbean genotypes using GGTT2 primer at an annealing temperature of 40°C. The lanes marked 1-22 corresponds to the genotypes numbered as 47 to 68 in **Table 1**.

of genotypes (63), while the GGTT2 primer identified the fewest genotypes (13). The CA1 primer, although showing a high number of markers, i.e., 21, could only identify 39 genotypes. The trinucleotide ISSR primer, CAA1 and GAT1, could identify 34 and 21 genotypes, respectively. Among the tetranucleotide ISSR primers, GGTT5 and GACA1 could identify 52 and 53 genotypes, respectively. The combination of profiles obtained using any two of the eight primers could identify a minimum of 63 genotypes (GGTT2 and GAT1) and a maximum of 87 genotypes (GA1 and GA5, GA1 and CAA1, GA1 and GAT, GACA1 and GA1). Rp of the primers (**Table 2**) ranged from 3.45 to 8.02.

DISCUSSION

In the present study, 87 genotypes were used that included 23 each from BARC and IARI, and the remainder were from other national and international institutes. Previous studies involved analyses of varying and smaller numbers of genotypes (for instance, the number of genotypes used were 46 by Saini *et al*. 2004; 30 by Reddy *et al.* 2008; 54 by Lavanya *et al*. 2008; 39 by Saini *et al.* 2010; 15 by Chattopadhyay *et al*. 2008; Dikshit *et al*. 2009 and Ullah *et al.* 2010; 17 by Tantasawat *et al*. 2010; 44 by Ratauri *et al*. 2011 and 16 by Taunk *et al*. 2012. Use of a larger number of genotypes will provide a better indication of the genetic

diversity existing in mungbean and yield a set of primers that may be appropriate for distinguishing genotypes.

From the results, it is obvious that optimising PCR conditions by ISSR primers have enhanced analysis capacity of mungbean genetic diversity.

Microsatellite sequence can be used as primer to get results, but they yield a large number of fragments that may be difficult to separate on agarose gels (Gupta *et al.* 1994). However, the study employed by one degenerate nucleotide of the two-base anchored primers that enclosed a lower specificity, thus producing a greater number of bands than would have been produced by non-degenerate, two-base anchored primers. A similar observation was reported in grapevine cultivars (Moreno *et al.* 1998). In the present study, the use of 3′ two nucleotide anchored primers ensured fewer products and as a result the PCR product analysis could be carried out on a 2% agarose gel. The extent of successful amplification by the ISSR primers designed in this study (24 of the 27) was higher than primers reported in the literature. For instance, in wheat only 33 out of the 100 commercially available ISSR primers gave successful amplification (Nagaoka and Ogihara 1997). After screening 46 commercially available primers, 11 gave amplification products in orange (Fang *et al.* 1998) and 4 primers produced distinguishable amplicons in citrus cultivars (Fang and Roose 1997). Raina *et al.* (2001) reported that only 29 of the 100 commercially available ISSR primers produced amplified fragments in *Arachis hypogea*.

Optimization of PCR conditions

In this study, PCR optimization was carried out in two steps mainly to obtain a clear band profile and as well as to obtain a larger number of bands that were polymorphic. This was accomplished by using six genotypes viz. TARM-18, RUM-5, JL-781, KOPERGOAN, PDM-116 and ML-5 obtained from different institutions in India (**Table 3**). Thus, using 8 ISSR primers, 135 polymorphic bands out of 145 amplified fragments were obtained in mungbean. Optimisation of the annealing temperature for ISSR primers to obtain better banding profiles has also been reported in wheat (Nagaoka and Ogihara 1997), rice (Blair *et al.* 1999), tea (Mondal 2002), *Brassica* (Martin and Sanchez 2000), *Citrus* (Sankar and Moore 2001), *Pandorea* (Jain *et al.* 1999) and grapevine (Moreno *et al.* 1998).

The PCR optimization for a multi-loci marker system by methods other than altering the annealing temperature has been reported in the literature such as a) by varying the concentration of additives such as formamide, ammonium sulphate, magnesium chloride, glycerol, etc. (Nagaoka and Ogihara 1997; Jain *et al.* 1999; Sankar and Moore 2001; Mondal 2002); b) by altering the number of PCR amplification cycles (Nagaoka and Ogihara 1997; Mondal 2002) and c) varying the concentration of template DNA (Jain *et al.* 1999; Mondal 2002). In the present study, a good number of polymorphic markers (**Table 2**) was obtained with 8 primers by optimization of the annealing temperature, hence other parameters were not carried out.

Characteristics of band profile

Polymorphism observed with marker is dependent on the number and diversity of the genotypes used in the analysis. In the current investigation, 135 polymorphic bands (93.1%) out of 145 amplified fragments was obtained from the 87 genotypes analyzed by using eight ISSR primers (**Table 2**). Even after optimizing the annealing temperature, 18.12 bands/primer obtained in this study which is higher than reported earlier by others for mungbean viz. 12.7 bands/primer by Lakhanpaul *et al.* (2000), 9.3 bands/primer by Saini *et al.* (2004) and 6 by Afzal *et al.* (2004). However, the higher number of bands/primer reported by Dikshit *et al.* (2007) and Kaga *et al.* (1996) could not be compared with the present study since another species belonging to subgenus *Ceratotropis* was included in addition to *V. radiata*. The level of polymorphism detected in the present study (93.1%) is higher than that reported previously by others. The percent polymorphism per primer reported is 64 by Lakhanpaul *et al.* (2000), 39.08 by Saini *et al.* (2004), 70 by Chattopadhyay *et al.* (2005), 92.9 by Saini *et al.* (2010). Although Lavanya *et al.* (2008) reported 24.85 bands/primer, this could not be compared with others as neither the percent polymorphism nor the number of polymorphic bands/primer was mentioned in the report. Among all the reports, the ISSR primers from present study had the highest number of polymorphic bands/primer (16.87) in addition to more bands/primer as well as a higher percentage of polymorphic bands. A higher level of polymorphism for ISSR compared to RAPD markers were reported in chickpea (Ratnaparkhe *et al*. 1998), wheat (Nagaoki and Ogihara 1997), *Pandorea* (Jain *et al.* 1999), finger millet (Salimath *et al.*1995) and sorghum (Oliveira *et al.*1996). Differences among the level of polymorphism in RAPD and ISSR fingerprints may be because of the number and nature of the fragments that get amplified. RAPD markers cover the entire genome, revealing length polymorphism in coding or non-coding and repeated or single copy sequences while ISSR markers are the product of amplification of the genome where the SSRs are present in large numbers. It is known that microsatellites have a higher mutation rate than the rest of the genome (Weber and Wang 1993). In addition to the above, optimization of annealing temperature and the use of large number of genotypes could have contributed to the better performance of ISSR primers.

Reproducibility

In the current study, the amplification profile was found to be highly reproducible as no variations were observed among the bands for a genotype-ISSR primer combination for different PCR runs and also for PCR amplification from different independent DNA preparations for the same genotypes. Some of the factors that could have contributed to the reproducibility of ISSR markers are use of primers in the range of 18-20 nucleotides in length, and use of optimized PCR annealing temperature for each of the primers which are in the range of 40 to 55°C that are lower than the T_m of the primers (60 to 70°C).

Genetic diversity

Knowledge about the genetic diversity among the genotypes of a germplasm allows a better organization and utilization of the crop's gene pool. Also, to protect breeders' rights, it is important to identify the cultivars. Often morphological characterization is the first step in plant identification, description and classification of the crop germplasm.

Genetic diversity analyses among Indian mungbean based on morphological and isozymes have been reported by Lakhanpaul *et al.* (2000) and Ghafoor *et al.* (2002). A number of studies in mungbean have utilised DNA markers to study genetic diversity.

The dendrogram obtained from the present ISSR analysis showed clustering of the genotypes into 5 groups. One major observation from the present study was that the genotypes developed at the two major institutes of India (BARC and IARI) shows two cluster groups that branches from the same node (**Fig. 4**). The genetic closeness among the BARC genotypes can be explained by the high degree of closeness among their pedigrees (**Table 1**). For instance, among the TARM series the TARM-1, TARM-2 and TARM-13 clustered together with one of the parent TPM-1. Similarly, E-series genotypes clustered close to one of the parents TARM-2. Also, among the nine genotypes that were selected for early maturing types (**Table 1**), 6 clustered genotypes together in Group IB, while, 10 out of 11 late maturing types (**Table 1**) clustered in Group IA (**Fig. 4**). Thus, based on ISSR markers, 20 out of the 23 genotypes developed at BARC clustered in Group I and a clear dis-

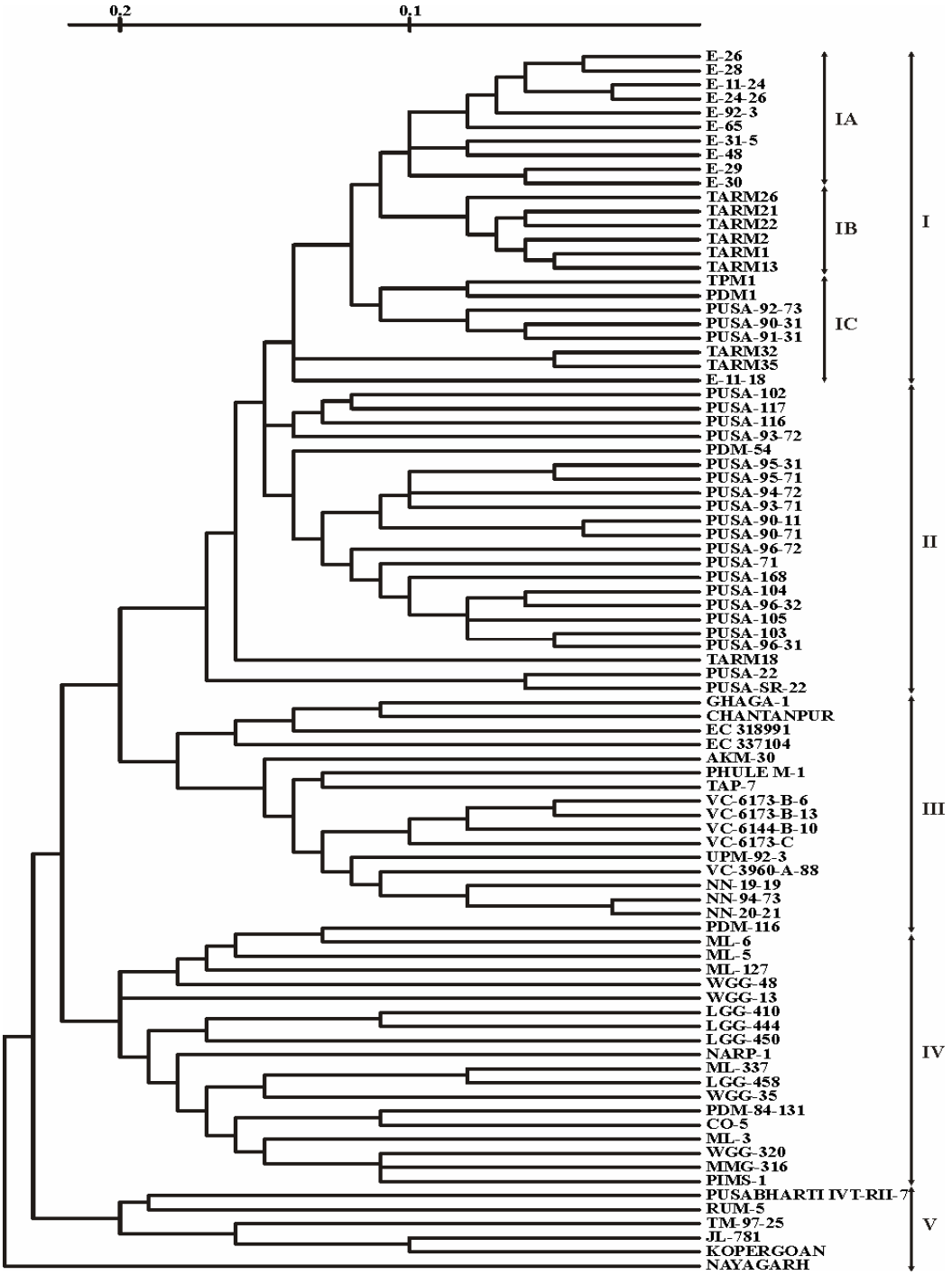

Fig. 4 Dendrogram showing the genetic relatedness among the mungbean genotypes derived from UPMGA cluster analysis.

tinction among the early and late maturing types of BARC genotypes was revealed.

The exact pedigrees of genotypes developed at IARI are unavailable, but they have been developed from exotic lines of mungbean from selections having common characters like high seed weight, high productivity, resistant to pests, etc. The possible reason for the genotypes developed at both the centers to cluster in two groups branching from the same node (**Fig. 4**) could be because of the use of at least one common parent used at BARC and IARI. TARM-18 was one of the TARM series that clustered in the Group II. The clustering of PDM-54 and TARM-18 (early maturing type) with the IARI genotypes may indicate them to have a common ancestor as that of Trombay genotypes.

Of the 23 genotypes developed at IARI, 20 clustered in the Group II. The PUSA-103 and PUSA-9631 genotypes are sister cultivars used by Lakhanpaul *et al.* (2000) also clustered and kept in the same group is evident from the dendrogram obtained in the present study. However, three of the genotypes PUSA-9273, PUSA-9031 and PUSA-9131 were clustered in a different group (**Fig. 4**, Group IC). Based on RAPD markers, similar observations were also reported for PUSA-9131 that clustered in a separate group (Lakhanpaul *et al.* 2000).

Saini *et al.* (2004) analysed 46 genotypes and divided them into two Groups (I and II). By AP-PCR analyses, they also observed similar relationships as found in the present study viz. a narrow genetic diversity among the genotypes developed at the two major Indian institutes (BARC, IARI) indicates that materials from these two locations were exchanged and genotypes were developed to suit widely different agro-climatic conditions. The general topology of the genetic diversity dendrogram among 46 genotypes from both these studies were same with a very few exceptions such as the placement of PUSA-90-11 and PUSA-90-71 as well as PUSA-22 and PUSA-SR-22. Clustering of the genotypes developed by an institute indicating a narrow genetic diversity, was also observed in this crop by several other

workers which involved different sets of genotypes and marker systems (Santalla *et al.* 1998; Lakhanpaul *et al.* 2000; Afzal *et al.* 2004; Saini *et al.* 2004; Reddy *et al.* 2008).

The third group was comprised of genotypes developed in India and its neighboring countries like Taiwan, Pakistan, etc. Among these genotypes, one Indian genotype UPM-92-3 developed at GB Pant Agriculture University, clustered with genotypes developed from Bangladesh and Pakistan. This observation is also indicative of the low level of genetic diversity among the mungbean genotypes possibly because of similar pedigree. However, TAP-7 and PUSA-103, despite having a similar ancestor, clustered into different groups. The reason for grouping in separate clusters may be because TAP-7 is a direct selection of the S-8 mutant, whereas PUSA-103 is a selection from a cross between the S-8 mutant and Cj-4; it is possible that Cj-4 is a diverse genotype and PUSA-103 is a selection that is closer to Cj-4.

The fourth group mainly consists of genotypes developed at different Indian states like Punjab, Andhra Pradesh, Tamil Naidu, etc. The result indicates that the type of cropping system or the agro-climatic conditions has no effect on the genetic diversity among the genotypes. Lakanpaul *et al.* (2000) also reported that close genetic similarity between the cultivars used in their study could be due to high degree of commonness between the pedigrees and apparently the crop cultivation system has apparently no effect on the genetic diversity; however, such reports on lower genetic diversity estimates using morpho-agronomic traits are not available.

The fifth group contains genotypes that are relatively more diverse than the remaining genotypes and were considered to be old selections of mungbean. Only TM-97-25 is an exception as it has been developed from a cross between KOPERGOAN and TARM-2 with selection toward KOPERGOAN type. Also, TARM-2 was developed from a cross between TPM-1 and RUM-5. As a result, this genotype might have major portion of the genome coming from KOPERGOAN and some part from RUM-5 and hence clustered in the last group.

Taking together that a large number of genotypes analysed were common between the present study and the study carried out by Saini *et al.* (2004) based on AP-PCR, indicates the suitability of ISSR for estimating genetic diversity. Similar observations have been made by other researchers for analyzing genetic diversity in other crop plants (Salimath *et al.* 1995; Nagaoka and Ogihara 1997; Ranade and Gopalakrishna 2001). In general, analysis of the dendrogram revealed that the genotypes developed from an Institute show a higher degree of similarity than other cultivars and such results have been obtained by others based on different marker analyses such as RAPD by Lakhanpaul *et al.* (2000). There results based on cluster analysis and PCA substantiate that greater homology existed between cultivars released from the same source. Bhat *et al.* (2005) studies on 27 cultivars revealed that very low genetic diversity exists between varieties released from the same Research Institute, possibly due to repeated use of same materials for mungbean breeding program. Also the AFLP cluster pattern for most of the variety was similar to RAPD cluster pattern obtained by Lakhanpaul *et al.* (2000).

Genotype identification

Identification of genotypes by using molecular techniques needs at least one difference in the banding pattern among the two genotypes under consideration. Mohanty *et al.* (2001) used albumins and globulins for identification of mungbean cultivars. Among the DNA markers ISSR based identification of genotypes is reported. For instance, three 5′ anchored primers together could distinguish 20 cultivars of *Brassica napus* (Charters *et al.* 1996); any 2 primers out of 10 were sufficient to distinguish all 37 accessions of *Lupinus albus* (Gilbert *et al.* 1999), etc. In the present study, designing and optimization of the primer annealing temperature identified eight primers that yielded a large number of

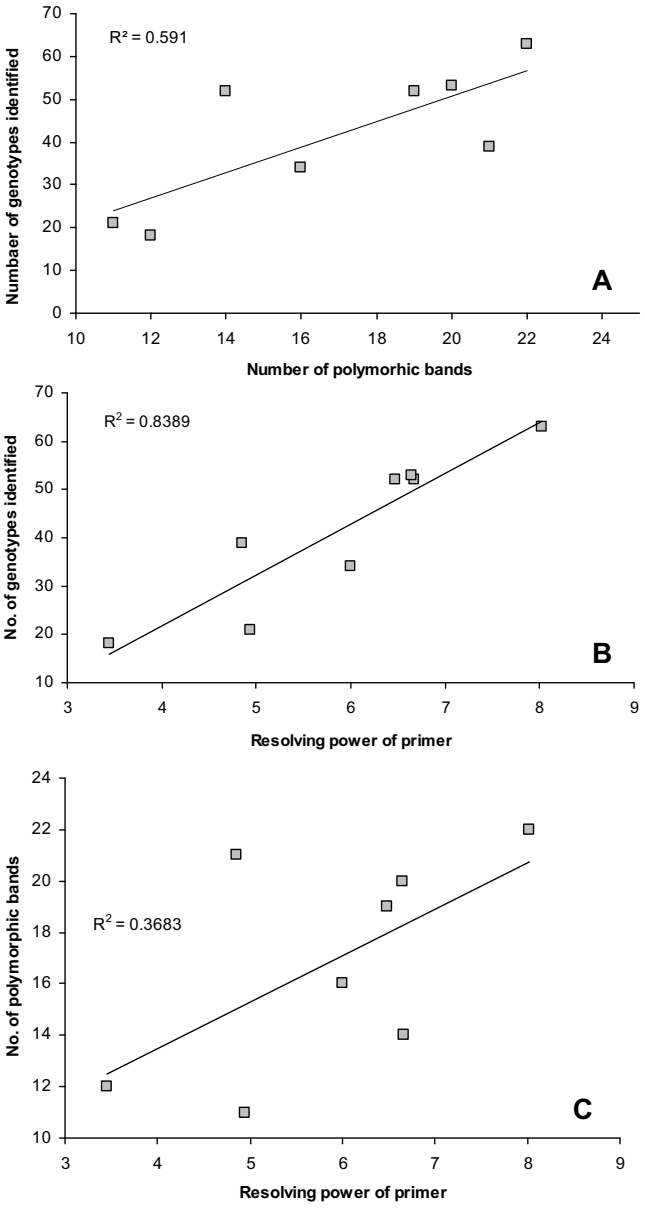

Fig. 5 Relationship between number of polymorphic bands, resolving power of a primer and number of genotypes identified by a primer. A genotype was considered identified provided at least one difference in the banding pattern among the set of genotypes. (**A**) Relationship between number of polymorphic bands (ISSR) and number of genotypes identified. (**B**) Relationship between Resolving Power of primers and number of genotypes identified. (**C**) Relationship between Resolving Power of a primer and number of genotypes identified by the profile generated by the primer.

polymorphic bands (**Table 2**). Although Dikshit *et al.* (2009) utilized Rp of RAPD primers as an indicative of identifying mungbean genotypes, we analysed the relationships between the number of genotypes identified with the number of polymorphic bands, resolving power of primer(s) (**Fig. 5**). Between the two, the resolving power of a primer showed a higher correlation with the number of genotypes identified (**Fig. 5B**; R = 9159; $P = 0.0014$) than the number of polymorphic bands with the number of genotypes identified (**Fig. 5A**; R = 7688; $P = 0.0258$). As expected from the above results, the number of polymorphic bands showed a lower correlation with the resolving power of primers (**Fig. 5C**; R = 607; $P = 0.11$).

In the present study, based on the relationship observed between Rp and the number of genotypes identified, it could be predicted that primers yielding an Rp value above 10 could identify all 87 genotypes. These results also suggest that the combined use of 8 ISSR primers with 135

polymorphic bands could allow all the genotypes to be distinguished up to a sample of 400 genotypes provided that the expanded set of genotypes has a similar level of genetic diversity to those included in the study.

CONCLUSIONS

The optimization of annealing temperatures led to increased efficiency of ISSR primers in analyzing genetic diversity of mungbean genotypes. The genetic diversity analyses clustered the genotypes into five groups. The clusters consisted of genotypes developed at a) BARC, b) IARI, c) genotypes from Indian and neighboring countries, d) genotypes developed in India and e) very old selections of mungbean. Thus the genetic relationships among the genotypes of *Vigna radiata* based on ISSR analysis could be useful for selecting parents for generating population for breeding purposes and genome mapping. The results presented in this paper show that ISSR primers are very efficient in the analyses of genetic diversity among *V. radiata* germplasm and to use them for the purpose of distinguishing the genotypes.

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