academicJournals

Vol. 12(47), pp. 6574-6582, 20 November, 2013
DOI: 10.5897/AJB11.2882
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

A comparative genetic diversity analysis in mungbean (*Vigna radiata* L.) using inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP)

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Accepted 7 December, 2011

Amplified fragment length polymorphism (AFLP) and inter-simple sequence repeat (ISSR) markers were used to study the DNA polymorphism in elite mungbean genotypes. A total of nine AFLP primer combination and 22 ISSR primers were used. Amplification of genomic DNA of the 30 genotypes, using AFLP analysis, yielded 300 fragments that could be scored, of which 192 were polymorphic, with an average of 21.3 polymorphic fragments per primer. Number of amplified fragments with AFLP primers ranged from 29 (E-AAC: M-CAG) to 10 (E-ACG: M-CAT). Percentage polymorphism ranged from 46.3% (E-AAC: M-CCA) to a maximum of 100% (E-AAC: M-CAC), with an average of 64%. The 22 ISSR primers used in the study produced 108 bands across 30 genotypes, of which 68 were polymorphic. The number of amplified bands varied from two UBC820) to ten URP 6F). The average numbers of bands per primer and polymorphic bands per primer were 4.9 and 3.1, respectively. Percentage polymorphism ranged from 25% (UBC844) to 85% (UBC846, UBC864, UBC895), with an average percentage polymorphism of 58.3% across all the genotypes. AFLP markers were more efficient than the ISSR assay, as they detected 64% polymorphic DNA markers in *Vigna radiata* as compared to 58.3% for ISSR markers. The Mantel test between the two Jaccard's similarity matrices gave r = 0.19, showing low correlation between AFLP- and ISSR-based similarities. Clustering of genotypes within groups was not similar when AFLP and ISSR derived dendrograms were compared.

Key words: AFLP, ISSR, *Vigna radiata* (mung bean), marker index, unweighted pair-group method with arithmetic averages (UPGMA).

INTRODUCTION

Mungbean is a widely grown food grain legume in the developing world. It is cultivated almost in all the four seasons in one state or the other state of India. It is thought to have originated in the Indian subcontinent (de Condolle, 1884; Vavilov, 1926) with maximum diversity in the upper Western Ghats and Deccan hills and secondary centre of diversity in Indo-Gangetic plains. From the standpoint of production, (19.7 mt), field pea(10.4 mt), chickpea (9.7 mt), cowpea (5.7 mt), lentil (3.6 mt) and pigeon pea (3.5 mt) are the most important

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(FAO, 2010). Out of the 16 essential nutrients, seven are classified as micronutrients or trace elements. Out of these seven micronutrients, iron and zinc play very important and vital role in animals, humans and plants health and development. Beside this, it also improves the soil fertility by fixing atmospheric nitrogen into available form with the help of *Rhizobia* species present in the nodules of its roots (Ashraf et al., 2003). The major constraint in pulses improvement is presence of limited genetic diversity in primary gene pool. In pulses, the morphological characterization of accessions belonging to cultivated species reveals only ample genetic variability for a trait other than genetic diversity. The problem of narrow genetic base of the cultivated germplasm of pulses develop from using only few genotypes with a high degree of relatedness repeatedly as parents in crossing programmes for the development of new cultivars (Kumar et al., 2011). Pedigree analysis of released varieties of mungbean revealed that only the top three to ten ancestors contributed 30 to 79% to the genetic base (Katiyar et al., 2007, 2008).

Therefore, an assessment of the genetic diversity of pulses is an important first step in a program to improve crop yield along with the traditional molecular makers which provide valuable information and can be used in a number of ways in crop improvement programme. Inter simple sequence repeat (ISSR) analysis involves the polymerase chain reaction (PCR) amplification of regions between adjacent, inversely oriented microsatellites, using a single simple sequence repeat (SSR) motifs (di-, tri-, tetra-, or penta-nucleotides) containing primers anchored at the 3′ or 5′ end by two to four arbitrary, often degenerate nucleotides (Souframanien and Gopalakrishnan, 2004). The primers used in our analysis were anchored at 3′ end to ensure that perfect annealing of the primer occurs at the 3′ end of the microsatellite motif, thus obviating internal priming and smear formation. The anchor also allows only a subset of the targeted inter-repeat regions to be amplified, thereby reducing the high number of PCR. The sequence of repeats and anchored nucleotides were randomly selected and had the advantage of analysing multiple loci in a single reaction. Thus, ISSR offers several advantages and this technique is already used in many crop plants (Ajibade et al., 2000; Ranade et al., 2000).

Further, AFLP (amplified fragment length polymorphism) which combines the advantage of RFLP and PCR, has a high multiplex ratio (number of loci observed in a single assay) and have high reproducibility and thus prove very useful in genetic diversity analysis. AFLP's were extensively used in genetic characterization of germplasm/ cultivars of black gram, soybean, wild bean etc. (Gupta and Gopalakrishna, 2009). Different molecular marker combinations were previously used in grain legumes (Gupta and Gopalakrishna, 2008, 2009). Therefore, the objective of the present study was to evaluate and compare the genetic diversity among 30 elite genotypes selected on basis of their performance in previous year experiments of known origin, using ISSR and AFLP markers.

MATERIALS AND METHODS

Plant material and DNA extraction

Table 1 lists the green gram genotypes used in the present study. Materials were collected from pulses station, CCS HAU, Hisar, India. The material was selected on the basis of contrast micronutrient content and agronomic performance in experiment conducted in the previous years (work un-published). Young leaves from three to five weeks-old seedlings were immediately stored at -80°C until the total genomic DNA was extracted. The molecular analysis was carried out at the laboratory of plant breeding, plant research international, Wageningen University.

The stored leaf tissue from each individual was ground to a fine powder using two grinding beads in a Shatter-box and total genomic DNA was extracted using 96 well plate automated DNA isolation machine. In the buffer solution, RNAse and proteinase K were added to get DNA free from these impurities. The estimates were confirmed by ethidium bromide staining of the gels after electrophoresis in 0.8% agarose gel at 100 V for about 45 min in TBE (Tris boric acid ethylene diamine tetra-acetic acid) buffer using known DNA concentration standards.

PCR optimization and primer selection

Varying concentrations of template DNA (10 to 20 ng), Taq DNA polymerase (0.5 to 2 U) and $MgCl_2$ salt (0 to 5 mM) were used to optimize reaction conditions of the polymerase chain reaction (PCR). Four randomly selected cultivars, namely ML-803, MH-125, ML-5, ML-735, were chosen for primer survey. Out of the different primers combination, only nine of AFLP and twenty two of ISSR were found suitable and used to analyse all 30 genotypes.

AFLP amplification

Li-Cor AFLP kit was used according to manufacturer recommenddations. According to the kit, 100 ng of pure DNA was digested with EcoR I and MSe I restriction enzymes. The enzyme adaptors were ligated to the digested DNA. The pre-amplification product was analysed on 1% agarose gel in 0.5XTBE buffer, run at 80 to 100 V for about 35 to 45 min. Selective amplification of restriction fragments was conducted using primers with three selective nucleotide labelled IRD700/800 with dyes.

After this, the reaction product was mixed with an equal volume 10 µl of formamide –loading buffer (98% formamide, 10 mM EDTA, pH 8.0 and 0.1% Bromo-phenol blue). The total mixture was carefully vortexed and heated for 5 min at 94°C in denaturation hot-block and then quickly cooled to ice). Out of the total 10 µl, 8 µl is loaded on a 6% denaturing polyacrylamide gel 1XTBE buffer. Li-Cor 4300 S DNA analyser machine was used to image, analyse and screen markers.

ISSR amplification

ISSR amplification reactions were carried out in 25-µl volume containing 50 ng template DNA, 0.5 U Taq DNA polymerase, 10

Table 1. List of mungbean (*Vigna radiata* L.) along with their origin, and micronutrients (Fe and Zn) used in the study.

Code No.	Variety	Origin	Code No.	Variety	Origin
1	ML-803	Ludhiana (Punjab)	16	ML-818	Ludhiana (Punjab)
2	MH-125	Hissar (Haryana)	17	ML-406	Ludhiana (Punjab)
3	ML-5	Ludhiana (Punjab)	18	2KM-151	Pant Nagar (Uttrakand)
4	ML-735	Ludhiana (Punjab)	19	2KM 155	-
5	2KM 112	IARI (New Delhi)	20	2KM-138	Hissar (Haryana)
6	ML-1108	Ludhiana (Punjab)	21	MH3-18	Hissar (Haryana)
7	MI-3580	Ludhiana (Punjab)	22	MH-124	Hissar (Haryana)
8	ML-839	Ludhiana (Punjab)	23	ASHA	Hissar (Haryana)
9	L-24-2	Ludhiana (Punjab)	24	MH-215	Hissar (Haryana)
10	MH-421	Hissar (Haryana)	25	SMH-99-DULL B	Hissar (Haryana)
11	2KM-139	Hissar (Haryana)	26	PDM-9-249	Kanpur (Uttar Pradesh)
12	2KM 135	(Rajasthan	27	ML-759	Ludhiana (Punjab)
13	SMH-99-2	Hissar (Haryana)	28	M 395	Ludhiana (Punjab)
14	2 KM-107	-	29	PMB-14	Ludhiana (Punjab)
15	BG-39	Bangladesh	30	ML-506	Ludhiana (Punjab)

mM dNTP, 10 µM primer, in 1× reaction buffer that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$. Amplification was performed in an Eppendorf Master cycler gradient. Amplification conditions were one cycle at 94°C for 4 min, and 94°C for 30 s, 55°C for 45 s, followed by 72°C for 2 min. In the subsequent 35 cycles, annealing temperature was maintained at 50°C, followed by one cycle of 7 min at 72°C. Amplified products were loaded on 2% agarose gel and separated in 1× TBE buffer at 75 V. The gels were visualized under UV after staining with ethidium bromide and documented using gel documentation.

Statistical procedure

Gel images were scored using the QUANTAR software for the presence (1) and absence (0) of an amplification product across the lanes for each of the primers combinations. Data were statistically analysed by the software program NTSYSpc 2.01b (Numerical Taxonomy and Multivariate Analysis System, Applied Biostatistics Inc., 1986 to 1997) (Rohlf, 1990). The SIMQUAL program was used to calculate the Jaccard's coefficient, a common estimator of genetic identity and was calculated as follows:

Jaccard's coefficient = $N_{AB} / (N_{AB} + N_A + N_B)$

Where N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A, and N_B represents fragments in sample B. Similarity matrices based on these indices were calculated. Similarity matrices were utilized to construct the un-weighted pair group method with arithmetic average (UPGMA) dendrogram. The Jaccard's similarity coefficients matrix was subjected to principal coordinates analysis for three-dimensional plot to depict the relationships among the varieties.

In order to characterize the capacity of each primer to detect polymorphic loci among the genotypes, marker index for AFLP and ISSR markers were calculated. It is the sum total of the polymorphism information content (PIC) values of all the markers produced by a particular primer. PIC value was calculated as follows:

PIC = *1-Σpi 2,*

Where, *pi* is the frequency of the i^{th} allele (Smith et al., 1997). While marker index (MI) is calculated as PIC value multiples with the number of polymorphic bands.

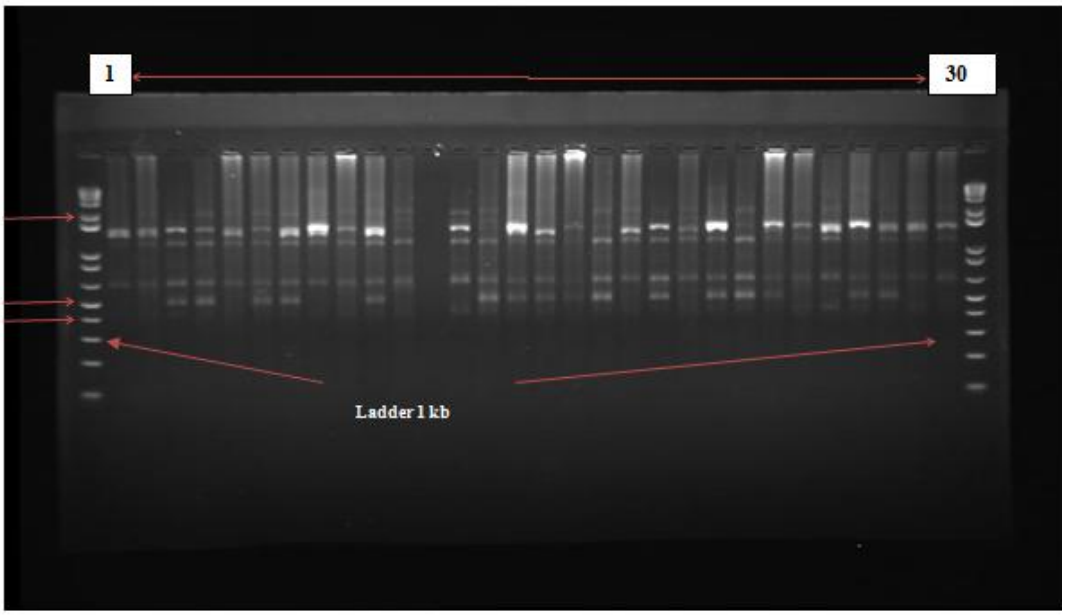
RESULTS

The criteria for selecting the primer pairs were a) high-quality of amplification, b) polymorphism among the cultivars used for primer survey. Figures 1a and b are representatives of AFLP and ISSR pattern obtained in the present study.

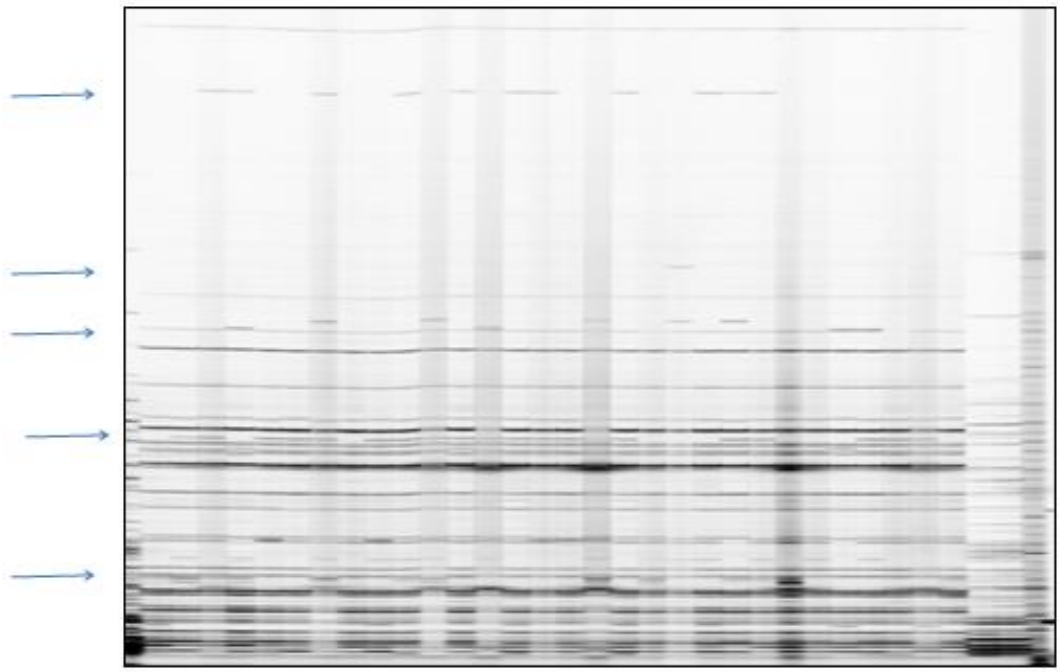
ISSR analysis

All the 23 ISSR markers were successfully amplified across the 30 mungbean genotypes but only 22 markers were polymorphic (Table 2). The 22 makers collectively yielded 108 amplification products with about bands bands per primer. ISSR primers gave 58.3% polymorphism and the PIC value ranged from 0.09 to 0.71 with an average of 0.46 (Figure 2a).

The UPGMA distributed the 30 genotypes into five main clusters, clusters with Dice similarity coefficient ranging from 0.65 to 0.85 (Figure 3a). Cluster I comprised of six genotypes (ML-803, MH-125, BG-39, 2KM 155, MH3-18 and MH-215).



a



b

Figure 1. (a) PCR amplification pattern for ISSR markers (UBC 821) in thirty green gram genotypes (Lane 1 and 32 is marker λ DNA marker and Lanes 2 to 31 green gram genotypes as listed in Table 1). (b) AFLP fingerprint generated by AFLP pair E-ACG:M-CAT in thirty green gram genotypes. The arrows indicate the polymorphic marker fragments.

Cluster II is the largest group among all the clusters with 12 genotypes (ML-5, ASHA, PDM-9-249, MH-124, SMH-99-DULL B, PMB-14, ML-506, MH-421, ML-406, 2KM-139, 2 KM-107 and 2KM-151) while cluster III comprised of only six (ML-1108, ML-759, MI- 3580, ML-839, M 395 and M 395) genotypes. 2KM112 and L-24-2

Table 2. List of primers and their sequences along with some of the characteristics of the amplification products obtained by selected twenty two ISSR primers in thirty genotypes of mungbean (*V. radiata* L.).

S/No.	Marker	Primer sequence (ISSR)	Total no. of amplification product	No. of polymorphic product	Percentage of polymorphism	PIC value of primer
1	UBC820	GTGTGTGTGTGTGTGTC	2	1	50	0.458
2	UBC836	AGAGAGAGAGAGAGAGYA	6	4	66.6	0.085
3	UBC821	GTGTGTGTGTGTGTGTA	5	4	80	0.144
4	UBC844	CTCTCTCTCTCTCTCTRC	4	1	25	0.194
5	IS 61	GAGAGAGAGAGAGAGAT	4	2	50	0.511
6	IS 65	AGAGAGAGAGAGAGAGT	4	2	50	0.523
7	UBC811	GAGAGAGAGAGAGAGAC	3	1	33.3	0.689
8	IS 63	AGAGAGAGAGAGAGAGC	3	1	33.3	0.611
9	UBC849	GTGTGTGTGTGTGTGTYA	4	2	50	0.465
10	UBC855	ACACACACACACACACYT	3	1	33.3	0.548
11	UBC857	ACACACACACACACACYG	7	4	57.1	0.635
12	UBC848	CACACACACACACACARG	7	5	71.4	0.462
13	UBC846	GAGAGAGAGAGAGAGAA	5	4	85.7	0.464
14	UBC864	ATGATGATGATGATGATG	7	6	85.7	0.433
15	UBC880	GGAGAGGAGAGGAGA	6	4	66.6	0.327
16	UBC812	GAGAGAGAGAGAGAGAA	3	1	33.3	0.537
17	UBC862	AGCAGCAGCAGCAGCAGC	3	1	33.3	0.704
18	URP 6F	GGCAAGCTGGTGGGAGGTAC	10	8	80	0.594
19	UBC835	AGAGAGAGAGAGAGAGYC	5	4	96	0.525
20	UBC859	TGTGTGTGTGTGTGTGRC	4	2	50	0.421
21	UBC895	AGGTCGCGGCCGCNNNNNNAT	7	6	85.7	0.419
22	URP 13R	TACATCGCAAGTGACACACC	6	4	66.6	0.463
		Total	108	68	1283	10.21
		Average	4.91	3.09	58.31	0.464

formed the fourth cluster and cluster V comprised of three genotypes (ML-5, 2KM 135, and SMH-99-2).

One genotype namely, ML 818 forms an out-group by not falling in any cluster. Principal component analysis (PCA) also resolved the ML-803 and BG-39 as a separate unit in cluster analysis (Figure 4a).

AFLP analysis

Nine AFLP primer combinations used to evaluate the genetic diversity among the 30 mungbean genotypes produced 300 scorable amplification products (Table 3). The number of amplified fragment per primer combination ranged from 13 (E-ACG: M-CAT) to 54 (E-AAC: M-CCA) with an average of 33.3 fragments per primer combination. The maximum polymorphism was shown by E-AAC: M-CAC (100% polymorphic) while the

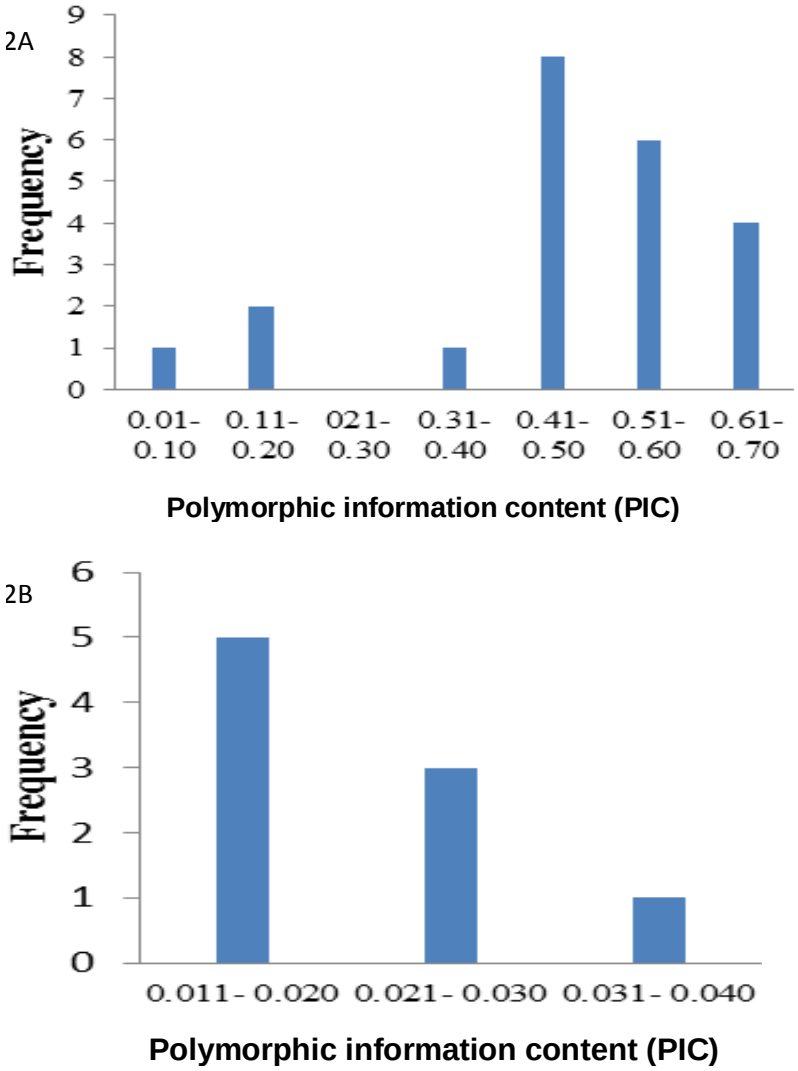


Figure 2. Polymorphic information content score for (a) 22 ISSR markers and (b) 9 AFLP primer combinations in thirty mungbean genotypes.

minimum polymorphism (46.3%) was shown by E-AAC: M-CCA. The PIC of individual AFLP markers varies from 0.01 to 0.04 (Figure 2b). Jaccard's similarity coefficient values were calculated from the data and Dice similarity coefficient ranged from 0.59 to 0.78 with an average of0.69. Further these were used to construct a UPGMA dendrogram (Figure 3b) and three-dimensional plot.

The UPGMA analysis distributed the 30 genotypes into main four clusters. Cluster I comprised of five genotypes (ML-803, BG-39, MH3-18, SMH-99-DULL B, PMB-14) and the cluster being the biggest cluster carried 16 genotypes (MH-125, 2KM 155, MH-215, ML-839, M 395, ML-5, 2KM 135, MH-124, MH-421, 2 KM-107, 2KM-151, 2KM 112, L-24-2, ML-1108, ML-759 and SMH-99-2).

Only five genotypes (MI-3580, 2KM-139, 2KM-138, ML-406, ML-506) were there in cluster III and genotypes, ML-735, PDM-9-249 and ASHA made the last cluster IV. In the AFLP dendrogram, ML-818 again makes an out-group as in case of ISSR dendrogram. Further from the PCA, it was found that ML-803 and Asha appeared to be distinct from other genotypes as in cluster analysis (Figure 4b).

DISCUSSION

Genetic variation was detected among 30 genotypes of mungbean using the ISSR and AFLP marker techniques. A total of nine primer combinations were used in AFLP study and all the primers gave polymorphic bands rang-

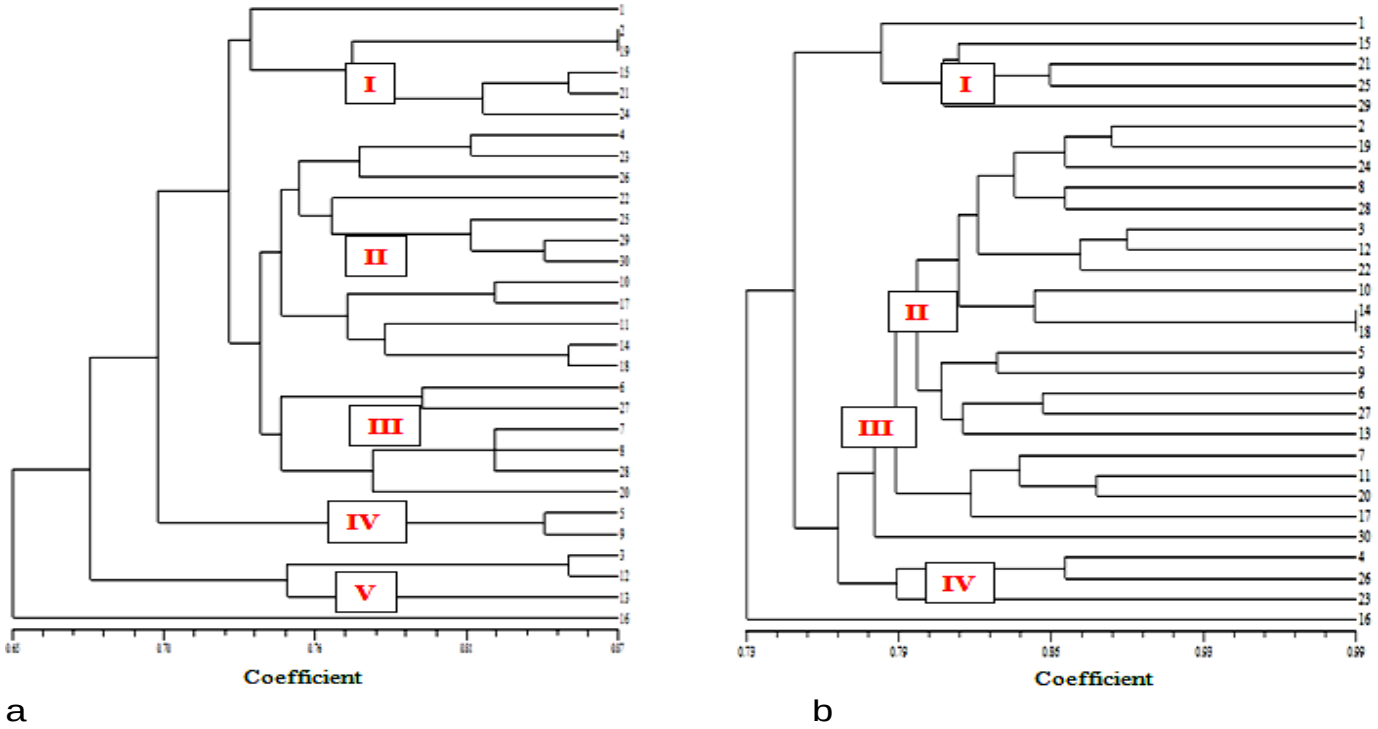


Figure 3. A UPGMA dendrogram of genetic relationships of the thirty genotypes constructed from (a) ISSR and (b) AFLP. ISSR, Inter-simple sequence repeat; AFLP, amplified fragment length polymorphism.

ing from 28 to 10 bands, while in case of ISSR markers out of the 23 primers only one did not show polymorphism while the rest showed. The results obtained in the present study shows a moderate genetic similarity for both the markers techniques (AFLP- 0.73 to 0.99; ISSR-0.65 to 0.87).

Thus, in comparison to the AFLP study ISSR responded well. This was also supported by previous studies (Russell et al., 1997; Meng and Chen, 2000). ISSR techniques have been previously used in estimating the genetic relationships in genus *Vigna* (Ajibade et al., 2000) and in several other crops (Souframanien and Gopalakrishna, 2004). Similarly, the AFLP marker techniques was also used successfully in different crops and legumes such as common bean (Tohme et al., 1996), cowpea (Coulibaly et al., 2002), pigeon pea (Panguluri et al., 2006), black gram (Gupta and Balakrishna, 2008) and azuki bean (Xu et al., 2000).

In the study, 22 polymorphic ISSR markers produced 68 alleles with an average 3.1 alleles per locus. The results are comparable with those reported earlier for other grain legume including black gram (3.4 alleles per locus; Souframanien and Gopalakrishna, 2004) and soybean (3.3 alleles per locus, Meng et al., 2001). The average PIC score of 33 ISSR markers was 0.46 with over 50% loci having PIC value > 0.5 (Figure 2a), indicating high resolving power of the ISSR markers. The markers were able to discriminate the 30 genotypes in five clusters on the basis of their performance in the field. Cluster I comprised of a short duration (<60 days), dwarf genotype with high resistance to mungbean yellow mosaic virus, while the biggest cluster II comprised of genotypes which showed moderate resistance against the MYMV virus and with medium height (65 to 70 cm). Cluster III comprise of early maturing tall (>85 cm) and moderate resistant genotypes while the smallest cluster V, which comprised of two genotypes with medium height, took average of 65 days to maturity and showed moderate resistance against the virus. As the marker is comparable to the traits shown by the genotypes in the field therefore, the ISSR markers thus would provide valuable tool for the varietal identification and germplasm maintenance in mungbean.

In AFLP, 9 AFLP primers combination generated 300 polymorphic bands with an average 21.3 bands per primer combination. As AFLP markers shows high multiplex ratios, much of the genome is covered using fewer primer combinations. Earlier the comparison of different marker techniques showed that the AFLP is the most efficient method to estimate genetic diversity compared to random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) (Powell et al., 1996;

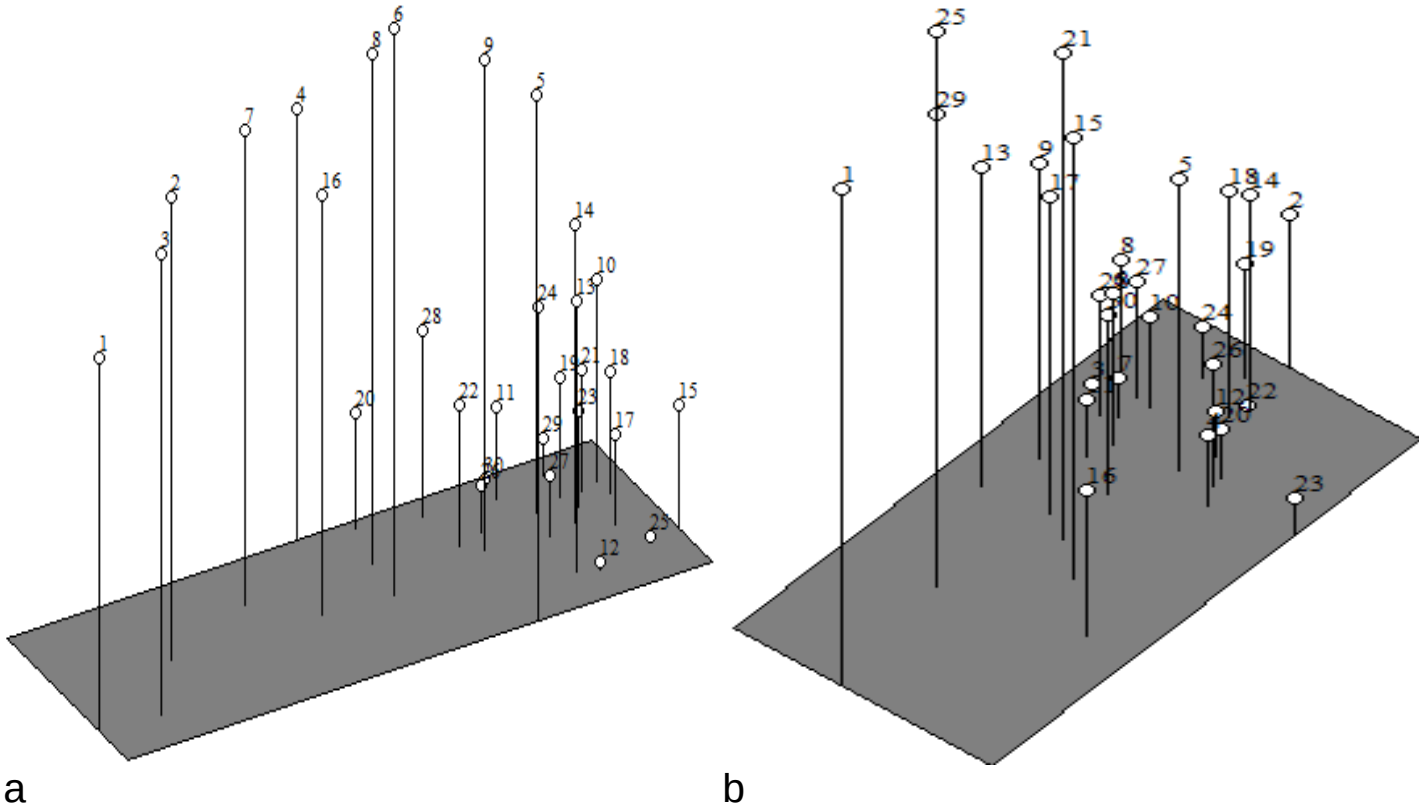


Figure 4. Principal componet analysis map showing the relationship among the mungbean genotypes based on (a) ISSR and (b) AFLP data. The number plotted represents individual genotypes and corresponds to those listed in Table 1. ISSR, Inter-simple sequence repeat; AFLP, amplified fragment length polymorphism.

Table 3. List of primers and their sequences along with some of the characteristics of the amplification products obtained by selected nine AFLP primer combination primers in 30 genotypes of mungbean (*V. radiata* L.).

S/N	Marker	Primer sequence (AFLP)	Total no. of amplification product	No. of polymorphic product	Percentage of polymorphism	PIC value of primer	Marker index
1	P32+P51	E-AAC:M-CCA	54	25	46.3	0.014	0.35
2	P35+P50	E-ACA:M-CAT	33	28	84.8	0.018	0.51
3	P35+P48	E-ACA:M-CAC	30	18	60.0	0.021	0.38
4	P37+P50	E-ACG:M-CAT	13	10	76.9	0.037	0.37
5	P35+P59	E-ACA:M-CTA	43	25	58.2	0.017	0.43
6	P35+P62	E-ACA:M-CTT	42	28	66.7	0.014	0.39
7	P32+P47	E-ACC:M-CAA	36	21	58.3	0.019	0.39
8	P32+P48	E-AAC:M-CAC	20	20	100	0.021	0.42
9	P32+P49	E-AAC:M-CAG	29	17	58.6	0.021	0.36
Total			300	192	609.8	0.182	34.9
Average			33.3	21.3	64	0.02	0.43

Gupta, 2008). To characterize the capacity of each primer to revel or detect polymorphic loci in the genotypes, primers index was calculated which varied from 0.35 (E-AAC:M-CCA) to 0.51 (E-ACA:M-CAT) with an average of 0.43. Cluster analysis from AFLP reveals that cluster II is the biggest cluster and carries mostly resis-

tance to moderate resistance with medium to tall genotypes while the smallest cluster III comprised of genotypes with early maturity (< 60 days) with medium height (65 to 75 cm) and with less or no resistance against the virus. The clustering pattern in both the markers system was comparable to some extent. In both the dendrogram, one genotypes ML-818 was out grouped which may be thought to be as a result of some contamination.

In the current study, the average PIC score for the ISSR marker (0.46) was much higher compared with AFLP marker (0.02) indicating that ISSR are more informative than the AFLP's. Cluster analysis based on the ISSR and AFLP data grouped 30 genotypes into six main clusters. However, clustering of the genotypes within the groups was not similar when both the dendrogram were compared. In PCA analysis, genotypes were resolved into four different groups in the case of the AFLP and results were congruent with the cluster analysis, but in case of ISSR, genotypes could not be resolved well in PCA.

The matrices for ISSR and AFLP marker were also compared using mantels test (Mantle, 1967) for matrix correspondence. The correlation values for the dendrogram based on ISSR and AFLP data was low (r = 0.19). A possible explanation to this low magnitude between the marker techniques is that the two marker techniques targeted different positions of the genome. The ability to resolve genetic variation among different genotypes may be more directly related to the number of polymorphism detected with each marker technique rather than a function of which technique is employed. Our results indicate the presence of moderate genetic variability among the elite mungbean genotypes. ISSR markers are useful in the assessment of the mungbean diversity and the selection of core collection to enhance the efficiency of germplasm management for use in the mungbean breeding and conservation.

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