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Microsatellite Markers Revealed Genetic Diversity in Mungbean Mutant Lines

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ABSTRACT

Estimation of genetic diversity in a crop species is prerequisite for its improvement. DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships precisely than other markers. Microsatellite markers are among the most popular genetic markers due to their characteristic features like high polymorphism, co-dominance, abundant informativeness, convenience of assay by PCR and distribution throughout the genome. The use of germplasm with distinct DNA profiles helps to generate breeding populations with broad genetic base. In the present study, SSR markers were used to analyze the genetic diversity of 30 mutant lines along with its parent PUSA-9072. A total of 8 primers were used for STMS analyses and 9 alleles were generated and the number of alleles per SSR primer ranged from 1 to 2 with an average of 1.15 per primer. The size of the amplification products varied in case of each primer and the range was 50bp to 250 bp. The dendrogram constructed based on SSR data using average linkage, grouped the mutant lines into two different clusters having all the mutants in one cluster and the parent in other. Clustering pattern based on SSR marker data indicated that there is a narrow genetic base of mungbean mutant lines. The absence of polymorphism indicates that the mutation were not on the tandem repeat region. The results indicate the usefulness of SSR in the assessment of genetic diversity in plants.

Keywords: Genetic diversity, SSR, Mungbean, Microsatellites, STMS

INTRODUCTION

Mungbean [*Vigna radiata* (L.) Wilczek] is one of the most important pulse crops, it is also known as greengram and is the most widely distributed among the six Asiatic *Vigna* species. Improvement of the cultivated plants largely depends on the extent of genetic variability available within the species. Besides natural genetic variation, mutagenesis is a potent device in creating variability and in obtaining novel traits (Sangsiri et al. 2005). Many mutants have made transitional impact on increasing yield and quality of several seed propagated crops (Ahloowalia et al. 2004). The mungbean variety, Pusa 9072 was treated ethyl methane sulphonate (EMS) and selected mutant lines were advanced to M_8 generation. Therefore, in the present study, an attempt has been made to study the induced variation among 30 mutant lines and parent genotype (Pusa 9072).

Conventionally, genetic diversity is estimated by morphological observations recorded on quantitative traits based on multivariate analysis, a potent tool in quantifying the degree of divergence in evaluation of germplasm collection of various crops. However, the results of such studies pertaining to genetic diversity are inconsistent; relevant only for genotypes used and environment involved in a particular study and cannot be generalized. Therefore, studies on above aspects on the available germplasm under specified environment where it is to be explained are essential for successful utilization of germplasm resources for the development of superior varieties.

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Advances in molecular biology have introduced an alternative for variety/genotype identification. The genetic characterization of germplasm helps in their effective conservation and reveals the extent of relationship among the accessions and the estimates of genetic diversity. The DNA of different individuals is tested for their similarity and dissimilarity at particular locus. Among the molecular markers, microsatellite markers are hyper-variable, co-dominant and highly reproducible, making them ideal for genome mapping (Jena et al. 2005), genetic diversity analysis (Brondani et al. 2005), varietal identification (Li et al. 2000), parentage analysis (Li et al. 2000; Yashitola et al. 2004), identification of heterozygotes and true hybridity (Nandakumar et al. 2004) and tagging and mapping of important traits (Sun et al. 2005). The most important uses of STMS markers are tagging gene for economic traits and for the study of genetic diversity.

MATERIALS AND METHODS

The mungbean genotype, Pusa 9072 was treated with a chemical mutagen, ethyl methane sulphonate (EMS) and 30 selected mungbean mutant lines were advanced to M_8 generation.

Extraction and purification of genomic DNA

The seeds of mungbean were sown in pots and kept in green house. After 8-10 days when the seedlings attained around 5-6 inches height, 15 plants were pooled and used for DNA isolation. Genomic DNA was isolated from mungbean seedlings using CTAB method (Saghai-Maroof et al. 1984) with minor modifications. Fresh and healthy seedlings were selected and frozen in liquid nitrogen and then crushed to fine powder using mortar and pestle. The fine powdered material was transferred to a preheated extraction buffer (20ml) in centrifuge tube. These mixtures were vortexed well and incubated in water bath at 600C for one hour with intermittent shaking. After incubation, equal amount of chloroform: isoamyl alcohol (24:1) was added to the centrifuge tube. Gently mixed the mixture and then centrifuged at 17000 rpm for 10 minutes in Sorval RC centrifuge at 25°C. The mixture was separated into two layers, *viz.* upper aqueous layer and lower organic layer after centrifugation. The aqueous layer was transferred into fresh centrifuge tube and DNA was precipitated by adding the equal amount of isopropanol or propanol. DNA was spooled out carefully with the help of dropper or wide bore tips and chemical was drained out. The precipitated DNA was transferred into 2ml eppendorf and washed 2-3 times with 70% alcohol. After washing the DNA was dried in vaccum drier. Dry DNA pellet was dissolved in minimum volume of Tris-EDTA (TE) buffer (10:1).

Extracted DNA were treated with RNase A (20mg/ml) at a concentration of 40 µl/ml of DNA and kept for incubation at 37°C for one hour. Protein k was added after one hour and kept for incubation again at 37°C for one hour. An equal amount of phenol: chloroform: isoamyl alcohol (25:24:1) was added after one hour to the solution and mixed by swirling for five minutes. After mixing, the solution was centrifuged at 10000 rpm for 5 minutes and upper aqueous layer was removed and transferred into fresh eppendorf tube. This process was repeated twice and DNA was precipitated by adding 1/10 volume of 3M sodium acetate (pH 6.5) and 2.5 times (v/v) chilled ethanol. Extra salt was removed by further washing with 70% ethanol and DNA was dried under vaccum. The dried DNA pellet was dissolved in T:E (10:1) buffer at room temperature and stored at 4°C.

Dilution of DNA

Purified and extracted DNA concentration was estimated using Hoefer DYNA Quant 200 Fluorimeter (Hoefer Scientific San Fransisco, USA) using Hoechst 33258 (Bisbenzamide) as the florescent dye and calf thymus DNA as the standard (Brunk et al. 1979). A part of DNA sample was diluted with appropriate amount of T:E (10:1) pH 8.0 to yield a working concentration of 20 ng/µl and stored at -20ºC.

Sequence Tagged Microsatellite Site analysis

Optimization of PCR component concentration was carried out for Taq DNA polymerase, $MgCl_2$, genomic DNA and primer. Concentration of dNTPs and 10X PCR buffer was not varied. PCR was carried out in a DNA thermal cycler (Gene Amp 9600 PCR system, Perkin Elmer Cetus Norwalk, CT, USA). The thermocycling conditions in PCR for microsatellite analysis were as follows, Denaturation for 94°C for 6 minutes, 30 cycles of denaturation at 94°C for 1 minute, primer annealing at respective annealing temperature for 1 minute

and primer extension at 72°C for 1 minute, final extension step at 72°C for 10 minutes and at 4°C till end.

A total of eight primers were selected for STMS analysis. The detail of used primers along with their sequences is given in table 1. The annealing temperature was standardized according to Tm of primer. Amplification reaction was performed in a final volume of 25 µl, consisting 1X reaction buffer (10mM HCL, pH 8.3, 50mm KCL), 0.25 µM of each primer, 2.0 mM MgSO4, 1U Ampli Taq DNA Polymerase, 60ng genomic DNA and 200 µM of each of dATP, dGTP, dCTP and dTTP. All the PCR amplification was done on 0.2ml Axygen thin walled PCR tubes. These tubes contain the above product were capped and spun at 15000 rpm for 2 seconds to allow proper settling of reaction mixture.

Table 1: List and Sequence of microsatellite markers used in the present study

Sl. No.	Primer	Primer sequence (5'-3')	Repeat Unit	Annealing temp.
1.	AB128079	AGGCGAGGTTTCGTTTCAAG	(AG)	55°C
		GCCCATATTTTTACGCCCAC		
2.	AB128093	CCCGATGAACGCTAATGCTG	(AG)	53°C
		CGCCAAAGGAAACGCAGAAC		
3.	AB128113	TCAGCAATCACTCATGTGGG	(AG)	55°C
		TGGGACAAACCTCATGGTTG		
4.	AB128135	AGGATTGTGGTTGGTGCATG	(AG)	55°C
		ACTATTTCCAACCTGCTGGG		
5.	VM22	GCGGGTAGTGTATACAATTTG	(AG)	52°C
		GTACTGTTCCATGGAAGATCT		
6.	VM24	TCAACAACACCTAGGAGCCAA	(AG)	48°C
		ATCGTGACCTAGTGCCCACC		
7.	VM31	CGCTCTTCGTTGATGGTTATG	(CT)	48°C
		GTGTTCTAGAGGGTGTGATGGT		
8.	MB122A	TGGTTGGTTGGTTCACAAGA	(TGGT)	48°C
		CACGGGTTCTGTCTCCAATA		

Gel electrophoresis

After completing the PCR amplification 2.0 µl of 6X loading dye (MBI Fermentas), was added. Mix the loading dye with PCR component and spun at 5000 rpm for 1 minute. Agarose (3.4%) gel in 1X TBE buffer with 10 µl ethidium bromide per 100 ml of gel volume was prepared. Electrophoresis was carried out at 50 volts for 2 hours. The gel was imaged with gel documentation system.

Data scoring and statistical analysis

Each amplification product was considered a DNA marker and was scored across all samples. The data on microsatellite were scored manually for the presence (1) and absence (0) of bands. Very faint bands were not considered for final scoring. The band sizes were estimated using 50bp to 1kb DNA molecular weight markers (MBI Fermentas). The data set of mutants and reproducible bands were used to calculate pair-wise similarity coefficients following Jaccard (1908). The matrix of similarity coefficients was subjected to unweighted pair-group analysis (UPGMA) to generate a dendrograms using average linkage procedure. The binary data were analysed using Free tree software.

RESULTS AND DISCUSSIONS

Mutation breeding is relatively quicker method for improvement of crops. Many physical and chemical mutagens have been used for induction of useful mutants in number of crops. However, the variability so induced may be in positive or negative direction. Genetic markers can be used to portray diversity within the cultivated germplasm and to identify grouping of cultivars which are adapted to particular regions (Paterson et al. 1991b). Earlier, assessment of genetic diversity has traditionally been made through morphological characters, which are often limited in number, have complex inheritance and vulnerable to environmental conditions. In present study, diversity induced through chemical mutagen (EMS) was studied in M_8 generation using SSR markers.

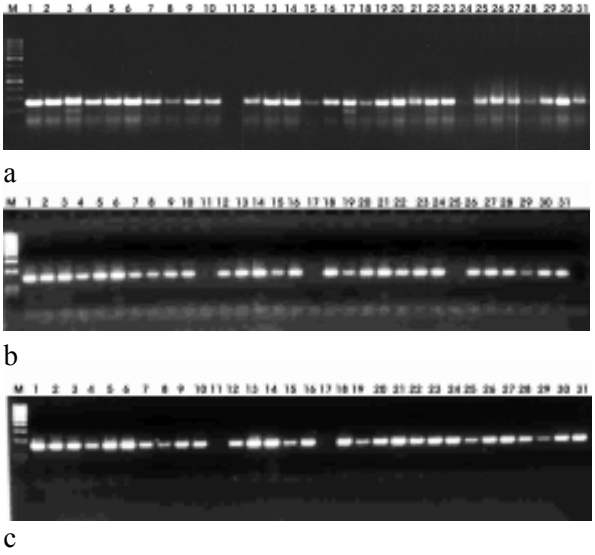
A total of 8 primers were used for STMS analyses and 9 alleles were generated and the number of alleles per SSR primer ranged from 1 to 2 with an average of 1.15 per primer (Table 2). Yu et al. (1999) reported the abundance and variation

Table 2: Characteristics of STMS amplification products generated by the eight STMS primer pairs in the mungbean mutants analysed

Sl. No.	STMS Primers	Total no of bands	No. of polymorphic bands	No. of banding pattern
1.	AB128079	01	00	01
2.	AB128093	01	00	01
3.	AB128113	01	00	01
4.	AB128135	01	00	01
5.	VM22	01	00	01
6.	VM24	01	00	01
7.	VM31	01	00	01
8.	MB122A	02	02	02

of microsatellite DNA sequences in *Phaseolus* and *Vigna* spp. Nasiri et al. (2009) has studied diversity in 77 genotypes of pea using 10 microsatellite markers and detected 59 alleles and number of alleles per locus varied from 2 to 8, with a mean of 5.9 alleles per locus. SSR markers are also utilized to decipher diversity in non legume crops like sorghum. Rajarajan and Ganesamurthy (2011) carried out genetic diversity analysis in 100 sorghum genotypes for drought tolerance using 13 stay-green specific polymorphic SSR markers which revealed high level of polymorphism among the genotypes, about 56 scorable alleles were generated, of which 55 were polymorphic and the number of alleles produced by different primers ranged from 2 to 7 with an average of 4.0 alleles per primer. However, Reddy et al. (2008) reported that accessions with most distinct DNA profiles are likely to contain the greatest number of novel alleles. It is these accessions that are likely to uncover the largest number of unique and potentially agronomic useful alleles.

The size of the amplification products varied in case of each primer and the range was 50bp to 250 bp. Fig. 1 is representative of the extent of polymorphism observed through STMS markers among the mutants. The numbers of banding pattern among the primers were also varied from 1 to 2. Cluster analysis of the distribution of STMS bands



a

b

c

Fig. 1: Banding pattern of mutant lines and parent revealed by SSR primer (a) AB128079, (b) AB128113 (c) AB128135. Lane M is standard marker 50 bp. Lane 1 to 30 mutant lines (AAIMM-1 to AAIMM-30) lane 31 is parent (Pusa 9072).

have been represented as a dendrogram using average linkage (Fig. 2). Similarity coefficient ranged for parent and mutants from 0.7778 to 1, while the mean value for parent was 0.7849 and for mutants 0.9857 (Table 3). The results of Xiao et al. (1996) involving crosses between four japonica and six indica elite inbred rice lines that are widely used in Chinese breeding programs, have indicated that genetic distance measures based on RAPDs and SSRs could be useful for predicting yield potential.

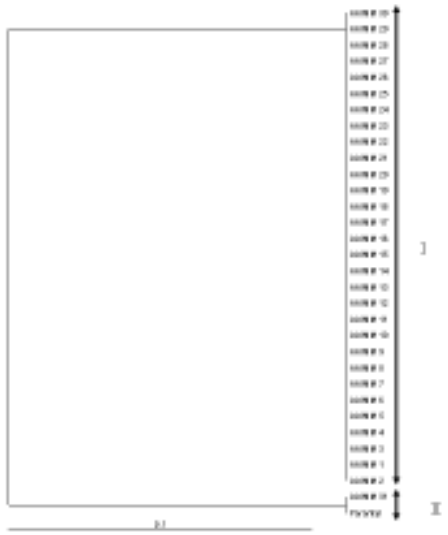


Fig. 2: Dendrogram revealed by UPGMA cluster analysis for 31 mutant lines and parent (Pusa-9072) based on STMS analysis

The dendrogram constructed based on SSR data using average linkage, mungbean mutants developed from chemical mutagenic treatment were grouped into two different clusters (Fig. 2). The cluster I consisted AAIMM-30, AAIMM-29, AAIMM-28, AAIMM-27, AAIMM-26, AAIMM-25, AAIMM-24, AAIMM-23, AAIMM-22, AAIMM-21, AAIMM-21, AAIMM-20, AAIMM-19, AAIMM-18, AAIMM-17, AAIMM-16, AAIMM-15, AAIMM-14, AAIMM-13, AAIMM-12, AAIMM-11, AAIMM-10, AAIMM-9, AAIMM-8, AAIMM-7, AAIMM-6, AAIMM-5, AAIMM-4, AAIMM-3, AAIMM-1 and AAIMM-2, while parent line AAIMM-31 was found in cluster II. Clustering pattern based on SSR marker data indicated that there is a narrow genetic base of mungbean mutant lines. Dikshit et al. (2007) found SSR marker was more efficient in detecting genetic variability among all *Vigna* species. But in the case

Table 3: Simple matching coefficients between the genotypes analysed based on STMS analysis

Mutant Lines	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1.00															
2	1	1.00														
3	1	1	1.00													
4	1	1	1	1.00												
5	1	1	1	1	1.00											
6	1	1	1	1	1	1.00										
7	1	1	1	1	1	1	1.00									
8	1	1	1	1	1	1	1	1.00								
9	1	1	1	1	1	1	1	1	1.00							
10	1	1	1	1	1	1	1	1	1	1.00						
11	1	1	1	1	1	1	1	1	1	1	1.00					
12	1	1	1	1	1	1	1	1	1	1	1	1.00				
13	1	1	1	1	1	1	1	1	1	1	1	1	1.00			
14	1	1	1	1	1	1	1	1	1	1	1	1	1	1.00		
15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.00	
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.00
17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
27	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
28	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
29	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
31 Parent	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778

*AAIMM-1 to AAIMM-31 are designated 1 to 31

Mutant Lines	17	18	19	20	21	22	23	24	25	26	27	28	29	30	Parent
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
12															
13															
14															
15															
16															
17	1.00														
18	1	1.00													
19	1	1	1.00												
20	1	1	1	1.00											
21	1	1	1	1	1.00										
22	1	1	1	1	1	1.00									
23	1	1	1	1	1	1	1.00								
24	1	1	1	1	1	1	1	1.00							
25	1	1	1	1	1	1	1	1	1.00						
26	1	1	1	1	1	1	1	1	1	1.00					
27	1	1	1	1	1	1	1	1	1	1	1.00				
28	1	1	1	1	1	1	1	1	1	1	1	1.00			
29	1	1	1	1	1	1	1	1	1	1	1	1	1.00		
30	1	1	1	1	1	1	1	1	1	1	1	1	1	1.00	
31 Parent	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	0.7778	1.00

of narrow genetic base of the *V. radiata* cultivars obtained a lower level of polymorphism in comparison to the RAPD and ISSR markers in mutants because the STMS markers are locus specific marker. The microsatellite markers are rapidly becoming a preferred type of DNA marker used for germplasm analysis and varietal identification, marker assisted selection and

genome mapping because it is locus specific marker but in the case of mutants' identification, very less variation was observed in the profile of mutants. Polymorphism is due to mutations in both the SSR region and the flanking regions contributing to the variation in allele size and number among different mutants.

CONCLUSIONS

The absence of polymorphism indicates that the mutations were not on the tandem repeat region. The SSR primer MB122A was identified as the best primer for mutant analysis and the SSR markers could be well utilized to analyze the mutants at DNA level.

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