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Assessment of Genetic Diversity Using ISSR Markers in Green Gram [*Vigna radiata* (L.) Wilczek]

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Molecular characterization is helpful in understanding the phylogenetic relationship among various germplasm to reveal the genetic diversity among the used parental genotypes. Among several efficient methods for revealing genetic variability within and among plant populations, one of the most widely applied methods is ISSR marker analysis. ISSR, markers are commonly used because they are quick, simple and environment non-sensitive enabling genetic diversity analysis in several types of plant material like natural populations, population in breeding programmes. Evaluation of genetic diversity would promote the efficient use of genetic variations, effective conservation and purity of the genotype to be determined as well as utilization of germplasm in crop improvement. ISSR marker analysis was performed to detect relatedness and diversity among eight parental genotypes. ISSR markers are useful in detecting polymorphism among accessions by generating a large number of markers that target multiple microsatellite loci distributed across the genome. Out of 109 scorable bands, 88 bands were polymorphic and the level of polymorphism was 81 per cent. Twenty five ISSR primers were used, out of which eighteen primers showed amplification in all genotypes. The average number of bands per primer was found to be 6.22 and average numbers of polymorphic bands per primer were 4.89. ISSR-01 proved to be best primer in the present investigation with total 29 fragments and eight highest scorable bands as well as 100 per cent polymorphism.

Introduction

Pulses offer one of the viable options for diversification of contemporary agriculture and management of natural resources. India is the largest producer and consumer of pulses in the world accounting 33 per cent of the area and 25 per cent of the global out-put. Green gram [*Vigna radiata* (L.) Wilczek) is the most important legume crop in India after chickpea and pigeonpea. It belongs to family Leguminaceae, subfamily Papillionaceae and its chromosome number is 2n = 2 x = 22. India is the primary green gram producer and contributes to about 75 per cent of the world pulses production. It contributes to about 14% of total pulses cultivation area and 7% of total pulses production in India. Green gram is extensively grown in India under varying soil types and climatic conditions and it improves soil fertility by fixing atmospheric nitrogen. It is a small herbaceous annual drought tolerant crop and suitable for dry land farming and predominantly used as intercrop with other crops. Being a short duration (60-65 days) crop with wide adaptability green gram grown

all over the world as a sole crop and as an inter crop or mixed crop with cereals. Besides being a rich source of protein, green gram enriches soil fertility through atmospheric nitrogen fixation with the help of *Rhizobium* bacteria in nodules and humus thus, plays a crucial role in furthering sustainable agriculture. For any successful breeding programme to improve grain yield and component characters, it is essential to know precisely the genetic architecture of these characters under prevailing conditions. Application of molecular markers to plant breeding has established the need for information on variation in DNA sequence even in those crops in which little classical genetic and cytogenetic information is available. Currently, the genetic diversity of plants has been assessed more efficiently after the introduction of the methods that reveal polymorphism directly at the DNA levels.

Materials and Methods

Final experimental trial comprising 8 parents along with 28 F_1s was evaluated during *kharif*, 2014 in randomized block design with three replications at RCA college farm, MPUAT, UDAIPUR. Eight diverse and well adapted genotypes of green gram were selected as parents for crossing programme, namely IPM-99-125, BM-4, ML-131, IPM 02-03, PDM-139, RMG-1035, RMG-344 and RMG-1045 (Table 1). All recommended cultural practices and plant protection measures were adopted to raise a good crop. Molecular analysis using ISSR markers was done exclusively for the parental material only. D Molecular marker analysis was done for the parental material to see the diversity present among the parental material. NA extracted from different green gram cultivars were compared using ISSR methodology. The leaves were harvested after 21 days and DNA was isolated with the help of Doyle and Doyle, 1987 protocol. DNA was extracted from young leaves (3–4 weeks old) using CTAB method and was amplified by using decamer random oligonucleotide primer in a DNA thermo cycler (Biometra).

For the ISSR reactions, 25 primer pairs were used. The DNA content in 20 µl of the reaction mixture was 50 ng. The sequences of these primers were purchased from Bangalore Genei Pvt. Ltd. The details of operon code sequence of the primer and G:C contents are given on table 3. The reaction contained 10X reaction buffer, 200 µM each of dNTPs ("Bangalore Genei"), 0.5 µM of each primer and 1 unit of Taq DNA polymerase (Table 2 and Fig. 1).

Submerged gel electrophoresis unit was used for fractionating amplified PCR products on 1.2% agarose gel. The gel was prepared in 1X TAE buffer containing 0.5 µg/ml of ethidium bromides. The samples and loading dye were mixed in 1:1 ratio and loaded with micropipette. In order to score and preserve banding patterns, photographs of the gel were taken by a Gel Documentation System, under UV transilluminator.

ISSR bands were designated on the basis of their molecular size ranging between 100-1000 bp. Electrophoresis was carried out at 100 V for 3 hr in 1X TAE electrophoresis buffer.

Gel was viewed under UV transilluminator and photographed by gel documentation system. Presence of amplified products was scored as 1 and its absence as 0 for all genotypes and primer combinations. These data matrices were then entered into NTSYS-PC developed by Rohlf (1993). The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationships of the genotypes using computer program NTSYSpc version 2.02.

Results and Discussion

Total genomic DNA was isolated with CTAB method Doyle and Doyle (1987). The powdered plant tissues extracted with extraction buffer containing chelating agent (EDTA) which helped to inactivate nucleases released from the plant cells which could cause serious degradation of the genomic DNA. The amount of DNA isolated from various genotypes of *V. radiata* L. ranged from 757 to 1518 ng/µl. The genotype IPM 02-03 yielded the highest amount of DNA (1518 ng/µl). Whereas the lowest amount of DNA (757 ng/µl) was obtained from genotype RMG-344.

The ratio of absorbance (A260/A280) ranged from 1.70 to 1.89 revealing that the DNA obtained was free from contaminants like polysaccharides, protein and RNA. The quality of DNA as also checked by gel electrophoresis revealed a single discrete band in all genotypes showing that genomic DNA was intact and had high molecular weight, free from any mechanical or enzymatic degradation, free from RNA contamination and was of high quality (Table 5).

Twenty five ISSR primers were used for the present investigation out of which eighteen primers showed amplification in all genotypes (Fig. 2). A total of 112 amplified bands were obtained from the 18 primers, out of which 88 were polymorphic. The total number of amplified bands varied between 5 and 8 (Table 3). The average number of bands per primer was found to be 6.22 and average numbers of polymorphic bands per primer were 4.89.

The polymorphism percentage ranged from 43 % (UBC-845) to 100% for five primers (ISSR-01, UBC-817, UBC-818, UBC-820 and UBC-854) used. Average polymorphism across all the genotypes of *V. radiata* L. was found to be 79%. Overall size of PCR amplified products ranged between 100 bp to 2000 bp. The PCR amplification using ISSR primers gave rise to reproducible amplification products. The number of potential ISSR markers depends on the variety and frequency of microsatellites, which tends to change with species (Table 6). Similar results were shown by Das *et al.*, (2014), Singh *et al.,* (2011) and Tantasawat *et al.,* (2010).

Genetic relationship and cluster tree analysis

The data obtained by using ISSR primers were used to construct similarity matrix of eight *V. radiata* L. genotypes using 'Simqual' sub-programme of software NTSYS-pc. Dendrograms were constructed using similarity matrix values as determined from ISSR data for *V. radiata* L. genotypes using unweighted pair group method with arithmetic average (UPGMA) sub-programme of NTSYS-pc software.

Similarity matrix

Based on ISSR similarity matrix data, the value of similarity coefficient ranged from 0.43 to 0.80 (Table 7). The average similarity across the eight parents was found out to be 0.62 showing that genotype were diverse from each other. Maximum similarity value of 0.80 was observed between genotypes RMG-1035 and RMG-1045 followed by RMG-1035 and RMG-344 with a similarity coefficient value of 0.79. Likewise, minimum similarity value of 0.43 was observed between genotypes BM-4 and IPM 02-03 and BM-4 with PDM-139 (44%). Similar findings were reported by Das *et al.,* (2014), Chattopadhyay *et al.,* (2005), Datta and Lal (2011) and Singh *et al.,* (2013) in green gram cultivars.

Table.1 Experimental material and their pedigree

Parent	Pedigree	Source
IPM 99-125	PM 3 x APM 36	IIPR, Kanpur
BM 4	MUTANT of T44	ARS, Badnapur
ML 131	ML 1 x ML 23	ARS, Durgapura
IPM 02-03	IPM 99-125 x Pusa bold 2	IIPR, Kanpur
PDM 139	ML 20/19 x ML 5	IIPR, Kanpur
RMG 1035	RMG 492 x ML 818	ARS, Durgapura
RMG 344	MOONG SEL.1 x J 45	ARS, Durgapura
RMG-1045	RMG-62 x KM 2170	ARS, Durgapura

Table.2 PCR reaction mixture content

Components	Final concentration	Single tube/20 (µl)
DNA template	50ng	2.00 µl
Master Mixture		
(i) dNTP MIX	200µM	1.6 µl
(ii) Taq polymerase	1 U	0.33µl
(iii) Reaction buffer (10x)	1X	2.00 µl
(iv) Primer	0.5 µM	1.00µl
(vi) dd H_2O		12.07µl

Table.3 List of ISSR primers

Sl No	Primer	Sequence (5'-3')	Annealing Temp. (oC)	No of bands (a)	No. of polymorphic bands (b)	Polymorphism % (b/a X 100)	Range of band size
1	ISSR-01	(GGC)5AT	67.2	8	8	100	100-1500
2	ISSR-02	(AAG)5GC	47.9	7	4	57	200-2000
3	ISSR-03	(AAG)5TC	45.5	NA	NA	NA	
4	ISSR-04	(AAG)5CC	47.9	5	3	60	100-700
5	ISSR-05	(AGC)5CA	57.6	7	6	86	200-2000
6	ISSR-06	(AGC)5CG	60	NA	NA	NA	
7	ISSR-07	(GGC)5TA	67.2	8	6	75	100-1500
8	ISSR-08	(AGC)5GA	57.6	8	7	88	100-1000
9	ISSR-09	(AAG)5CG	47.9	5	3	60	100-700
10	ISSR-33	(AG)8AT	51.4	NA	NA	NA	
11	UBC-810	(GA)8T	43.3	7	4	57	300-1000
12	UBC-811	(GA)8C	43.5	7	6	86	300-1000
13	UBC-813	(CT)8T	41.3	NA	NA	NA	
14	UBC-817	(CA)8A	45.8	5	5	100	200-600
15	UBC-818	(CA)8G	49.0	6	6	100	200-1000
16	UBC-820	(GT)8T	53.3	5	5	100	100-700
17	UBC-822	(TC)8A	51.9	7	5	71	100-1500
18	UBC-824	(TC)8G	43.3	NA	NA	NA	
19	UBC-836	(AG)8YA	45.0	5	4	80	300-900
20	UBC-840	(GA)8YT	43.3	NA	NA	NA	
21	UBC-845	(CT)8RG	43.5	7	3	43	200-600
22	UBC-848	(CA)8RG	41.3	5	4	80	300-1000
23	UBC-854	(TC)8RG	52.7	6	6	100	200-1500
24	UBC-873	(GATA)4	45.8	NA	NA	NA	
25	UBC-878	(GGC)5AT	49	4	3	75	500-2000
		Total		**112**	**88**	**79**	**-**
		Average		**6.22**	**4.89**	**-**	**-**

Table.4 PCR reaction cycle

Cycle	Denaturation		Annealing		Extension	
First cycle	94°C	5 min	-	-	-	-
2-35 Cycle	94°C	1 min	Tm (Pr)	45 sec	72 °C	2 min
Last cycle	-	-	-	-	72°C	10min

Table.5 Quality and quantity of total genomic DNA of *V. radiata* L. isolated and purified by CTAB method

Genotypes	Parents' Name	Concentration (ng/ µl)	Ratio 260/280
P1	IPM 99-125	1420	1.81
P2	BM-4	968	1.77
P3	ML-131	1250	1.79
P4	IPM 02-03	1518	1.89
P5	PDM-139	1251	1.80
P6	RMG-1035	1012	1.81
P7	RMG-344	757	1.74
P8	RMG-1045	998	1.82

Table.6 Details of the ISSR primers used for amplification of genomic DNA of green gram

Total number of primers	25
Number of primers which showed amplification	18
Number of primer which showed polymorphism	18
Total number of monomorphic bands	21
Total number of polymorphic bands	88
Total number of bands	109
Total number of amplicon produced	563

Table.7 Similarity matrix of green gram genotypes

	IPM 99-125	BM-4	ML-131	IPM 02-03	PDM-139	RMG-1035	RMG-344	RMG-1045
IPM 99-125	1.00							
BM-4	0.49	1.00						
ML-131	0.64	0.47	1.00					
IPM 02-03	0.49	0.43	0.54	1.00				
PDM-139	0.48	0.44	0.53	0.49	1.00			
RMG-1035	0.57	0.47	0.64	0.49	0.63	1.00		
RMG-344	0.60	0.49	0.66	0.51	0.66	0.79	1.00	
RMG-1045	0.60	0.52	0.58	0.48	0.61	0.80	0.74	1.00

Figure.1 Protocol used for ISSR primers for PCR amplification

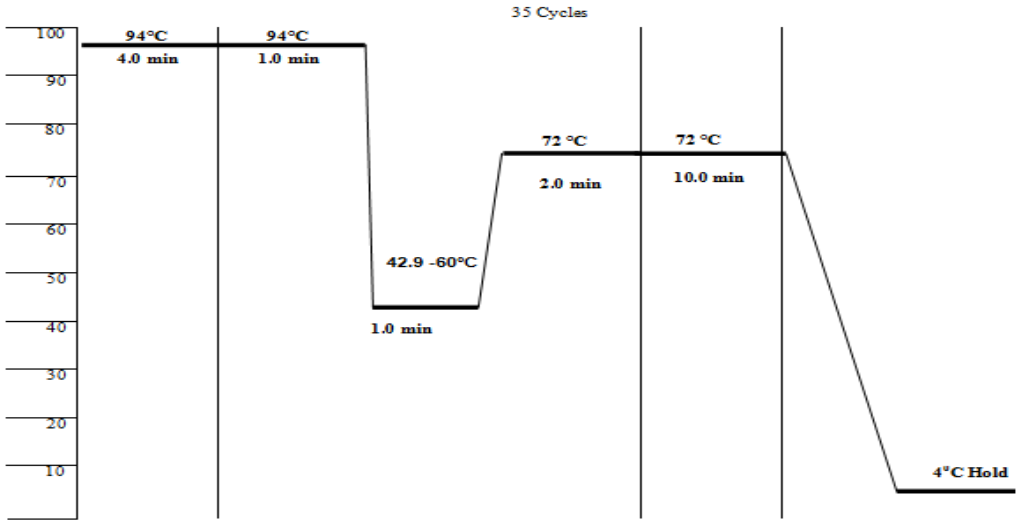


Figure.2 3d ISSR

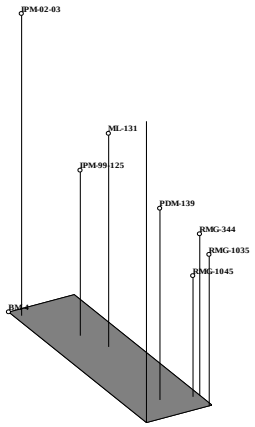
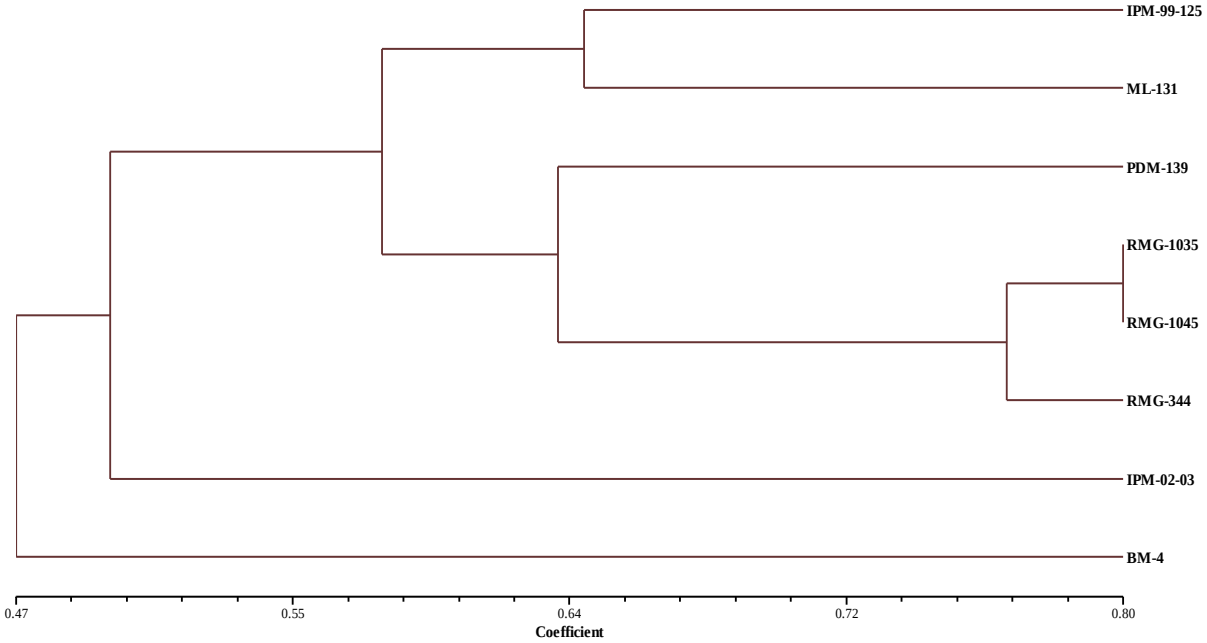


Figure.3 Dendrogram of greengram genotypes using ISSR markers



Cluster tree analysis

The ISSR data based derivation of similarity matrix shown in table 6 reveal the similarity values lay between 0.43-0.80. The dendrogram clearly indicated four major clusters (Fig. 3). Cluster I included two genotypes IPM 99-125 and ML-131 are similar to each other at a similarity coefficient of 0.64. Cluster II is the main one that included four genotypes *viz.*, PDM-139, RMG-1035, RMG-1045 and RMG-344. It could be divided into two, sub-clusters IIA which joined with sub cluster IIA at similarity coefficient of 0.62. Sub-cluster IIA included only one genotype viz. PDM-139. Sub cluster IIB included three genotypes and also divided as IIB 1 and IIB 2. IIB 1 has two genotypes

RMG-1035 and RMG-1045 are similar to each other at a similarity coefficient of 0.80, these two genotypes showed the maximum similarity coefficient. Subcluster BII 2 included only one genotype viz. RMG-344. Cluster III and cluster IV included only one genotype IPM 02-03 and BM-4 respectively. Cluster II joined with cluster IV at similarity coefficient of 0.49. Similar results have been reported by Singh *et al.,* (2013). The UPGMA distributed the 30 genotypes into five main clusters; similarity coefficient values ranging from 0.65 to 0.8. One genotype namely, ML 818 forms an out-group by not falling in any cluster. The genetic variation amongst advanced lines of diverse crosses, also found the same result by Tantasawat *et al.,* (2010) and Bharati *et al.,* (2012).

Molecular analysis through ISSR markers revealed that cross BM 4 and IPM 02-03 followed by BM-4 and PDM-139 has high parental genetic diversity having 57 per cent and 56 per cent dissimilarity respectively. Therefore, the hybrid of BM 4 x IPM 02-03 and BM-4 x PDM-139 turned out to be the most promising on the basis of its parental genetic diversity and high *per se* performance, in both for seed yield and its components. Therefore this cross can be gainfully utilized.

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