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Assessment of genetic variability in mutant lines of greengram (*Vigna radiata*) using ISSR markers

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Genetic improvement of crop plants largely depends on the extent of genetic variability available within the species. Besides natural genetic variation available in greengram [*Vigna radiata* (L.) Wilczek] germplasm collections, mutagenesis is proven to be a contemporary device in obtaining novel traits and creating genetic variability (Sangsiri *et al.* 2005). Many mutant varieties have made transitional impact on increasing yield and quality of several crop plants (Ahloowalia *et al*. 2004). The success of any mutation breeding largely depends on appropriate dose and application of mutagen and, as a result, extensive studies including a number of highly effective mutagens both physical and chemical are used (Singh and Sharma 1993, Yaqoob and Rashid 2001). Therefore, induced mutants in greengram would be quite useful in creating genetic variability for quantitative traits besides their use in fundamental studies.

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. It is well documented that the DNA markers have many advantages over the traditional morpho-biochemical markers. In recent years, isozymes, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and sequence tagged micro satellite (STMS) markers have been used extensively in various pulse crops. The analyses based on isozymes (Jaaska and Jaaska 1990), RAPD (Tomooka *et al*. 1996) and RFLP (Kaga *et al.* 1996) have confirmed that the adzuki bean group, greengram group and aconitifolia group are distinct. Genome relationship between greengram and cowpea [*V. unguiculata* (L.) Walp] based on the linkage arrangement of random genomic RFLP has been investigated by Menancio *et al.* (1993). Souframanien and Gopalakrishna (2004) were analysed the genetic diversity in blackgram by ISSR and RAPD markers and find the ISSR marker were more efficient than the RAPDs. In the present study, 31 mutant lines (in M8 generation) generated through ethyl methyl sulphonate (EMS) threatment of a high yielding greengram cultivar Pusa 9072 were subjected to diversity analysis based on agro-morphological traits and ISSR profile. The extent of genetic variability generated through mutagen treatment and the efficiency of ISSRs in diversity assessment have been discussed.

The experimental materials comprised 31 mutant lines (M8 generation) and one parental line (Table 1) of greengram cv Pusa 9072. The mutant lines were selected based on yielding ability through the generations after mutagenic treatment of cv Pusa 9072, a high yielding greengram cultivar with EMS.

The agro-morphological traits of mutant greengram lines were assessed through field trial at experimentation centre of Department of Genetics and Plant Breeding, Allahabad Agricultural Institute-Deemed University, Allahabad during *zaid* 2008 in a randomized block design with three replications and 30×10 cm spacing. Recommended cultural practices were followed to raise a healthy crop. The observations were recorded on 10 randomly selected plants from each replication of each genotype for days to 50% flowering, plant height, number of clusters/plant, pod length, number of seeds/pod, days to maturity, 100-seeds weight and seed yield/plant.

DNA was isolated from the leaf tissues using a CTAB based method (Saghai-Maroof *et al.* 1984) with minor modifications. The extracted DNA (~20 ng/μl) was used as template for polymerase chain reaction (PCR). A total of

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fourty primers were screened of which sixteen primers were selected for ISSR analysis on the basis of polymorphism and reproducibility of bands. Detail of annealing temperature of primers is given in Table 1. Individual PCR amplifications of each ISSR were carried out in a final volume of 25µl, consisting 1 X reaction buffer (10mM HCl, pH 8.3, 50mM KCl), 10.0 mM primer, 2.5 mM $MgCl_2$, 40 ng genomic DNA, 10.0 µM of dNTPs mixture and 1U *Taq* DNA Polymerase. PCR amplifications were carried out in a thermal cycler (Gene Amp 9600PCR system, Perkin Elmer USA) with cycling conditions of initial denaturation (94ºC) for 7 minutes, 30 cycles of denaturation at 94º for 30 seconds, respective annealing temperature (Table 1) for 45 seconds and 72ºC for 2 minutes, followed by a final extension step at 72ºC for 5 minutes. PCR Amplification were resolved in 1.4% TBE-agarose gels and photographed using a gel documentation system after staining with ethidium bromide.

The data on agro-morphological traits were subjected to analysis of variance and then mean values, standard errors (SE) and critical differences (CD) were determined. The mean values for all agro-morphological traits were standardized and analysed by cluster analysis (UPGMA and Euclidean distance) using NTSYS-pc v2.1 software. The data of ISSR were scored manually for the presence and absence of bands as 1 and 0, respectively. 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 method analysis (UPGMA) to generate a dendrograms using average linkage procedure. All the computation was performed using NTSYS-pc v2.1 software (Rohlf 1993).

Analysis of variance indicated significantly variation

Table 1 Agro-morphological traits of 31 mutant lines and Pusa 9072 (parent)

Line	Accessions No.	Days to 50% flowering	Plant height	No. of Cluster/ plant	Pod length	No. of seeds/ pod	Days to maturity	100 seed weight (g)	Seed yield/ plant (g)
1	AAIMM-1	39.33	30.05	7.65	4.05	9.13	62.50	2.80	7.52
2	AAIMM-2	38.33	53.40	11.68	6.66	10.98	60.00	3.67	10.25
3	AAIMM-3	37.33	49.30	13.38	5.48	11.10	65.50	3.23	19.63
4	AAIMM-4	35.00	46.73	18.20	7.08	10.70	64.00	3.70	16.83
5	AAIMM-5	39.67	40.10	16.60	6.55	10.70	63.00	3.88	14.83
6	AAIMM-6	34.33	63.20	14.76	6.62	10.50	64.50	3.72	25.57
7	AAIMM-7	34.33	60.53	13.38	7.39	12.10	63.00	3.38	20.14
8	AAIMM-8	38.00	49.09	19.33	7.69	12.00	59.50	4.40	30.35
9	AAIMM-9	34.67	57.37	13.80	6.97	12.30	62.00	3.65	26.12
10	AAIMM-10	41.00	54.60	12.90	7.02	10.00	60.00	4.21	33.08
11	AAIMM-11	39.33	60.22	19.20	7.91	10.90	66.50	3.50	20.33
12	AAIMM-12	39.67	54.00	20.94	7.71	12.10	66.00	5.25	31.20
13	AAIMM-13	35.00	47.73	15.33	7.19	10.60	65.00	3.76	25.10
14	AAIMM-14	38.00	45.97	16.15	7.24	12.90	62.50	3.63	19.64
15	AAIMM-15	33.00	33.40	11.10	6.20	8.40	68.00	3.78	13.44
16	AAIMM-16	40.93	40.93	12.17	7.53	10.95	65.50	3.35	16.50
17	AAIMM-17	46.43	46.43	12.18	6.72	9.85	69.50	3.46	14.43
18	AAIMM-18	43.73	43.73	13.20	6.84	10.20	65.00	3.61	18.71
91	AAIMM-19	38.13	38.13	15.38	6.62	9.70	63.50	3.84	15.12
20	AAIMM-20	42.90	42.90	13.60	6.46	10.50	65.50	3.25	25.84
21	AAIMM-21	44.15	44.15	17.20	7.21	11.70	64.00	3.95	17.95
22	AAIMM-22	37.45	37.45	12.70	6.23	10.00	63.00	3.34	23.08
23	AAIMM-23	35.05	35.05	15.69	6.69	9.65	62.00	3.90	12.15
24	AAIMM-24	39.20	39.20	12.80	7.00	10.00	58.50	4.00	20.52
25	AAIMM-25	42.42	42.42	15.53	6.81	12.80	69.00	3.55	25.35
26	AAIMM-26	43.27	43.27	15.35	6.82	10.70	64.00	3.70	23.26
27	AAIMM-27	47.57	47.57	12.70	6.46	9.10	63.00	3.40	16.84
28	AAIMM-28	49.53	49.53	16.80	6.35	11.90	66.00	3.42	30.33
29	AAIMM-29	37.37	37.37	11.98	6.25	9.25	65.00	3.78	20.23
30	AAIMM-30	44.80	44.80	13.70	6.36	9.80	65.00	3.16	26.09
31	AAIMM-31	41.83	41.83	14.00	6.58	10.90	64.50	3.24	25.00
	Mean	39.73	45.82	14.50	6.73	10.69	64.03	3.66	20.82
	SE	0.86	2.79	1.21	0.21	0.52	0.85	0.14	1.94
	CD (P=0.05)	2.44	7.90	3.48	0.61	1.48	2.41	0.40	5.50
	Pusa 9072	31.66	53.40	14.10	7.15	11.50	66.50	3.68	26.52

among the mutants for all the characters studied. The mean values of agro-morphological traits are given in Table 1. Mutant line AAIMM-4, AAIMM-8, AAIMM-11 and AAIMM-12 recorded significantly higher clusters/plant than the parent. Whereas, days to maturity has been reduced significantly in almost all the mutant lines except AAIMM-17 and AAIMM-25. A significant increase in 100-seed weight was found in AAIMM-8, AAIMM-10 and AAIMM-12. The yield/plant in the mutant lines was recorded to be at per or lower than that of the parent except AAIMM-10 which registered highest yield/plant.

Based on eight agronomic traits, all the mutants and parent (Pusa 9072) were grouped in single cluster with the ranged from 1.0285 to 8.7823 at 5.02 scales (Fig 1). Cluster-I was further divided in cluster Ia, cluster Ib, cluster Ic, cluster Id and cluster-Ie. Cluster Ia comprised a total of 13 mutant lines, whereas cluster Ib consisted only four mutant lines. Parent was clustered in cluster Ic with mutant AAIMM-6, AAIMM-7 and AAIMM-9. Mutant lines AAIMM-8, AAIMM-12 and AAIMM-11 were clustered in cluster Ie. The cluster Id consisted mutants AAIMM-14, AAIMM-21, AAIMM-25, AAIMM-28 and AAIMM-15. Mutant AAIMM-10 was recorded highest seed yield/plant while mutant AAIMM-1 the lowest yielder. Both the lines were distinct in dendrogram (Fig 1). Mutant AAIMM-24 was the early mature line which is also distinct in cluster diagram.

In the present study 16 ISSR primers (Table 2), among the 40 primers screened on a subset of five mutant lines, resulted in discrete profiles with all DNA tested. Sixteen ISSR primers resulted in consistent profiles and were used for all 31 mutant lines and parent. Among these primers, primer 23 gave the maximum number of polymorphic bands (5), followed by primer 1, 17, 21 and 826 (each amplified 4 polymorphic bands). A total of 163 amplification products were scored in 31 mutant lines and parent. Out of these amplification products the 42.89% were polymorphic with an average, 10.25 bands/primer. The number of DNA fragments amplified for a given primer combination varied from 6 to 13 (Table 2). Resolving power of gel was found to be maximum for primer 1 (2.84), followed by for primer 826 (2.64) and primer 23 (2.54). The fragments produced from 31 mutants and the parent line varied in size from 50 to 2500 bp, were used as input data for computation of pair-wise distances. The similarity coefficient ranged for mutants from 0.88 to 0.99 with a mean value of 0.92. The lowest distance (0.88) was noted between the mutant AAIMM-21 and AAIMM-22, and between AAIMM-31 and parent. Highest distance (0.99) was recorded between the mutant AAIMM-4 and AAIMM-5 and between AAIMM-5 and AAIMM-6. Low to moderate genetic diversity was observed between mutant lines resulted due to mutagenesis. The ISSR profiles of the mutant lines indicated that considerable variability can be generated through mutagenesis which provides scope for further exploitation through breeding.

The clustering pattern of the mutant lines and parental line based on ISSR profiles is given Fig 2. Mutant lines were grouped into two major clusters, cluster I (25 mutants) and cluster II (2 mutants and parent). Cluster I was further divided in two clusters, Ia and Ib. Cluster Ia consisted of 16 mutants, and cluster Ib consisted nine mutants. Mutant line AAIMM-30, AAIMM-31were clustered along with the parent in cluster II. The out group mutants, viz. AAIMM-22, AAIMM-9, AAIMM-23 and AAIMM-24 was clearly distinguished from all the mutant lines (Fig 2).

Clustering patterns of greengram mutant lines based on ISSR profile and morphological traits were found to be dissimilar to some extent. It is well documented that the molecular markers are more effective to determine the

Table 2 List of ISSR primers, sequence and characteristics of amplification products generated by the 16 primer in the greengram mutants analysed

Primer	Primer sequence (5'-3')	Annealing temperature (ºC)	Total No. of bands	No. of polymorphic bands	No. of banding pattern
Primer 1	$(CA)_6RY$	48	12	04	08
Primer 2	$(CA)_6RG$	48	12	01	02
Primer 3	$(CA)_6R$	48	11	02	04
Primer 5	$(AGC)_4Y$	55	10	03	04
Primer 14	$(AC)_8YT$	55	09	02	04
Primer 15	$BDB(CA)_7$	55	09	01	02
Primer 17	$VHV(GT)_7$	55	10	04	09
Primer 18	$HVH(TG)_7$	55	13	00	01
Primer 21	$(AC)_8T$	55	11	04	10
Primer 22	$(AG)_8T$	48	10	03	04
Primer 23	$(AG)_8C$	48	11	05	11
Primer 25	$(GA)_8T$	48	06	01	04
808	$(AG)_8C$	55	12	01	03
812	$G(AG)_7TT$	52	07	01	02
826	$A(CA)_7CC$	55	11	04	08
827	$A(CA)_7CG$	55	09	02	05

Y, Pyrimidine; R, Purine; B, Non-A(C,GorT); D, Non-C(A,GorT); H, non-G(A,CorT); V, Non-T(A,CorG)

variability in compared to morphological markers (Rana *et al.* 2005, Senior *et al.* 1998). However, the level of polymorphism is low in ISSR markers study in comparison to previous study it may due to the mutant lines were developed from single parent.

Comparison between morphological and molecular analysis both markers were useful in genetic analysis of mutants. The genetic variation at morphological level was further validated by molecular markers. The ISSR marker was found to be more suitable for diversity analysis. This study can be further extended using chromosome based SSR markers and analyzing their relationship with genetic variation in morphological traits. The present study showed that some of the ISSR markers like primer 23, primer 1, primer 17, primer 21 and 826 were most efficient primers in analyzing the variation among greengram mutant lines which can be used for genetic diversity analysis in greengram. Based on morphological characterization the mutant AAIMM-10 recorded to have high yield capacity and AAIMM-24 was an early maturity line. Further, all the

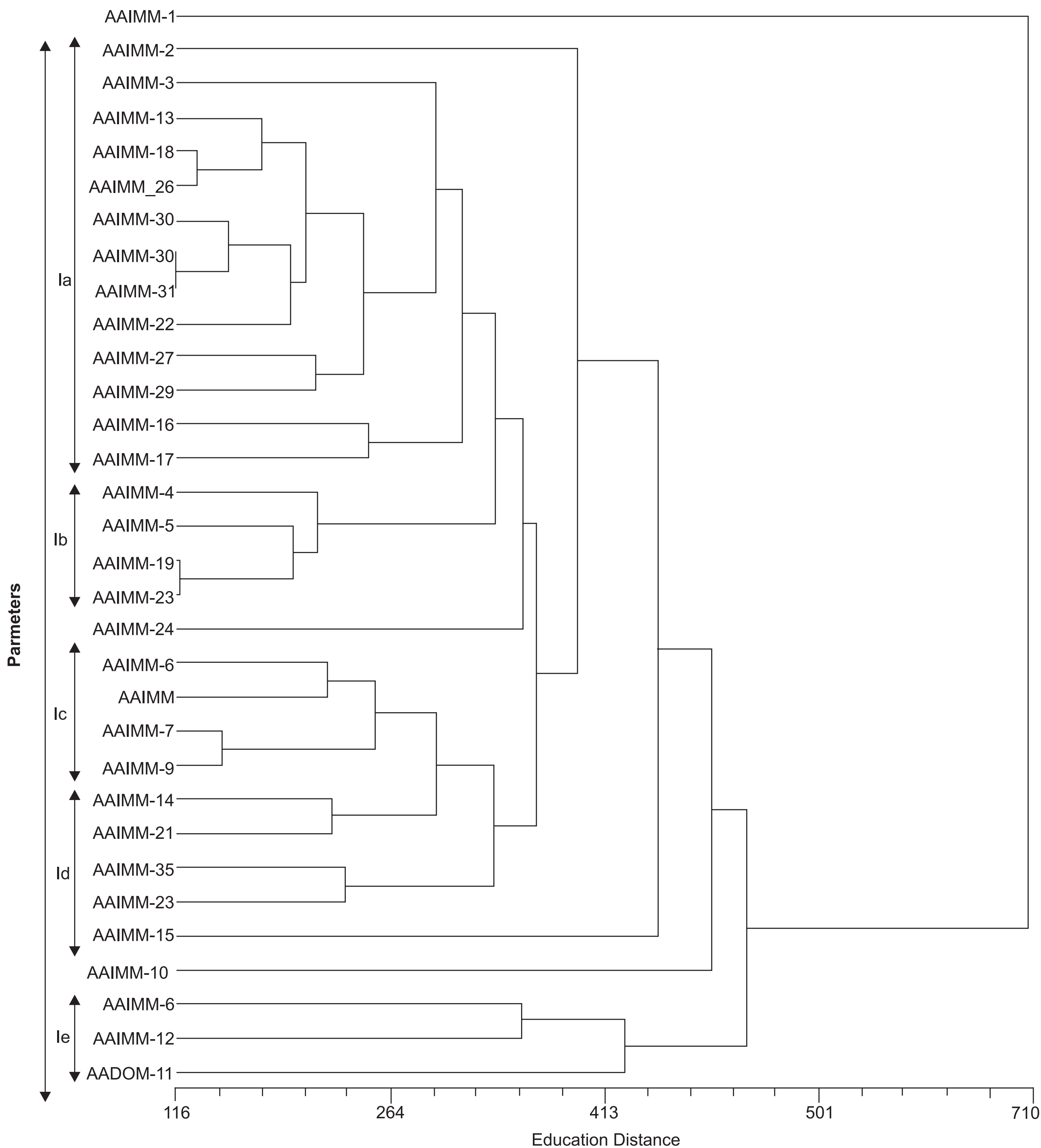

Fig 1 Agro-morphological diversity among the greemgram mutant lines and their parental line

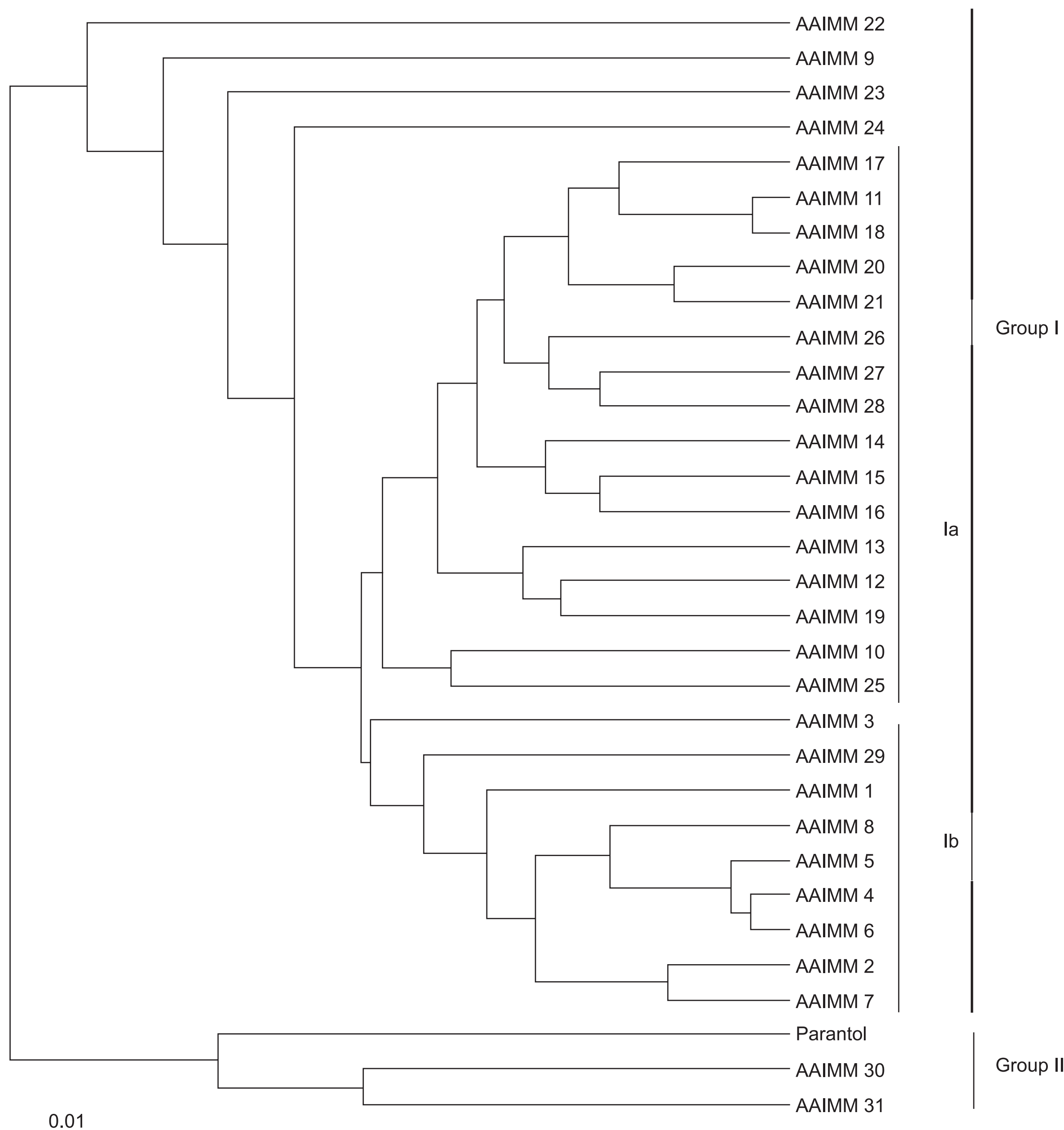

Fig 2 Clustering of greengram mutant lines and their parental line based on ISSR profile

desirable mutants are required to be evaluated in a larger trial at multi-locations along with the existing commercial varieties as control. The higher yielding mutants can be developed into varieties for commercial cultivation. Besides, the mutants with superiority in a specific character(s) can be used successfully in programme for breeding superior varieties.

SUMMARY

Out of 40 ISSR primers used in this study, 16 markers resulted in 163 amplifications with an average of 10.25 bands/ primer. Among 163 amplifications, 38 (42.89%) were found to be polymorphic. The genetic similarity coefficients among the mutant lines ranged from 0.88 to 0.99 with a mean value of 0.92. Cluster analysis based on ISSR profile grouped the genotypes into two major clusters which further divided into sub-clusters. The molecular diversity among the mutant lines was greater than that of the morphological diversity which also revealed that considerable amount of genetic variability has been generated through mutagenic treatment. The superiority of mutants AAIMM-10 and AAIMM-24 over the parental line in terms of yield and early maturity respectively proves the relevance of this study.

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