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SSR-marker Assisted Evaluation of Genetic Diversity in Greengram [*Vigna radiata* (L.) Wilcezk]

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ABSTRACT

Background: Assessing genetic diversity in greengram is a prerequisite for its genetic improvement for yield and quality. DNA markers such as simple sequence repeats (SSRs) have been preferred in this crop for the analysis of genetic diversity because SSR markers are locus-specific, widely dispersed throughout the genome, highly polymorphic due to variation in repeat units, highly informative because of co-dominant nature, high reproducibility and ease of assay by PCR (Polymerase chain reaction). The current study aimed to study the genetic diversity among the mutants of greengram at the molecular level using SSR markers.

Methods: In this study, twenty-five SSR markers were used to analyze the genetic diversity amongst twenty mutant genotypes of greengram along with their parents. Genomic DNA was isolated from the leaves by using the standard CTAB DNA extraction method. Then DNA purification and PCR amplifications were carried out. The genetic variability and diversity among genotypes was examined by assessing the scoring of the amplified bands by SSR -PCR amplification.

Result: Seventeen SSR primers generated 102 polymorphic bands with an average of the six polymorphic bands per primer. The number of alleles per locus ranged from four to nine. The size of amplification product varied for each primer and the range found to be 100bp to 2000bp. The mean polymorphic information content (PIC) value for SSR markers was found to be 0.6335. The value of Jaccard's similarity coefficient had ranged from 0.07-0.70 with an average value of 0.38. The dendrogram constructed on SSR molecular markers data through the unweighted pair group method with arithmetic averages (UPGMA) method had enabled grouping of the genotypes into thirteen clusters. The results indicate the usefulness of SSR markers in the assessment of genetic variability and diversity among the mutant genotypes of greengram.

Key words: Genetic diversity, Greengram, Molecular marker, Mutants, PIC, SSR, UPGMA.

INTRODUCTION

Greengram [*Vigna radiata* (L.) Wilczek], also known as goldengram, mungbean, mashbean and moong, is one of the most important pulse crops and belongs to the family *Fabaceae* (*Leguminosae*). It is a self-pollinated diploid species with a chromosome number of 2n = 22 and an estimated genome size of 579 Mbp (Arumuganathan and Earle, 1991). Based on domesticated diversity data and archeological evidence, the origin of greengram is considered to be India and during the early domestication process, its cultivation migrated to other Asian countries and to Africa, Australia, South America and other parts of the World (Fuller and Harvey, 2006; Kim *et al*., 2015; Vishnu-Mittre, 1974). There is great interest in genetic and genomic analyses of greengram due to this crop's valuable nutritional and health benefits, especially in developing countries, where malnutrition is a major issue. It has been known to be an excellent source of dietary fiber, high-quality protein, minerals, vitamins and significant amounts of bioactive compounds, including polysaccharides, polyphenols and peptides, therefore, becoming a popular functional food in promoting good health. The greengram has been documented to ameliorate hyperglycemia, hyperlipemia and hypertension and prevent cancer and melanogenesis, as well as possess hepatoprotective and immunomodulatory activities (Hou *et al*., 2019). This crop was selected by AVRDC-The World Vegetable Center as an iron-rich food source to fulfill nutritional needs (Vijayalakshmi *et al*., 2003). The low productivity of this crop can be attributed to a narrow genetic base resulting in low yield potential and susceptibility to biotic and abiotic stresses. The knowledge of genetic diversity has a significant impact on the improvement of crop plants and this information has been successfully used for efficient germplasm management, fingerprinting and genotype selection. The genetic diversity within the genotypes of mungbean based on the morphological trait is comparatively low because it is predominantly self-pollinating (Sudha, 2009). Recently DNA markers such as SSRs have been preferred in this crop for the analysis of genetic diversity because SSR markers are locus-specific, widely dispersed throughout the genome, highly polymorphic due to variation in repeat units, highly

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informative because of co-dominant nature, high reproducibility and ease of assay by PCR (Powell *et al*., 1996; Kuleung *et al*., 2004; Liu *et al*., 2013; Zia *et al*., 2014; Chen *et al*., 2015). The present study was thus undertaken to study the genetic diversity among mutants of greengram at the molecular level using SSR markers.

MATERIALS AND METHODS

Plant materials and DNA extraction

In the present investigation, seeds material of twenty superior productive mutants (Table 1) along with their parent Sujata and OBGG-52, obtained from the harvested material of M_5 generation were taken for SSR analysis. Seedlings were grown in pots and fresh young leaf samples were collected from 10-20 days old seedlings before sunrise and washed thoroughly in distilled water and stored at -20°C for further analysis. Total genomic DNA from the leaves was isolated by using the standard CTAB (cetyl trimethyl ammonium bromide) DNA extraction method (Doyle and Doyle, 1990).

Purification of DNA

For purification of DNA, extracted DNA was treated with RNAse-A at a concentration of 60 µg/ml of DNA and kept for incubation at 37°C for an hour with continuous shaking in the water bath. After one hour it was removed from the water bath and an equal volume of chloroform: isoamyl alcohol (24:l) was added and mixed thoroughly. The solution was then centrifuged at l0000 rpm for 10 minutes and the upper aqueous phase was pipetted out. Starting from the addition of chloroform-isoamyl alcohol, the entire process was repeated twice. For further purification, the DNA solution was washed with phenol:chloroform: isoamyl alcohol (25:24:l) and subsequently thrice with chloroform: isoamyl alcohol (24:l). The upper aqueous phase was separated after centrifugation as per the procedure described earlier and mixed with l/10th volume of 3M sodium acetate (pH 4.8). DNA was precipitated by adding 2.5 volumes of chilled absolute ethanol and into a pellet by centrifugation at 12000 rpm. The pellet was washed twice with 70% ethanol carefully and dried under vacuum. DNA quality was confirmed by electrophoresis on 0.8% agarose gel and quantified by using a UV-vis spectrophotometer. After quantification, the DNA was diluted in TE buffer (pH 8.0) to a working concentration of 25 ng/µl for PCR analysis and stored at -20°C.

SSR primer amplification

For SSR analysis, PCR amplifications were carried out in a total volume of 20 µl containing 50 ng of template DNA, 1 unit of *Taq* DNA polymerase, 0.25 mM each dNTP, 0.25 mM primer (each forward and reverse) in 1× PCR reaction buffer. Template DNA was initially pre-denaturated at 94°C for 3 min. followed by 35 cycles at 94°C for one min denaturation, primer annealing between 46-53°C (depending upon primer) for one min and extension at 72°C for one min. Finally, incubated at 72°C for ten minutes to complete the primer extension process. The obtained PCR products were used for electrophoresis on 1.5% agarose gels stained with ethidium bromide at 70 V for 3 hours and photographed under UV light using Gel Doc system (Genai image system, UVITech, UK). The gel doc photograph was used for documentation and scoring the bands.

Data analysis

The potential of the marker for estimating genetic variability and diversity was examined by assessing the marker informativeness through scoring the amplified bands by SSR -PCR amplification based on the presence (1) or absence (0) of bands for each primer. Primer banding characteristics such as the total number of bands, number of polymorphic bands and percentage of polymorphic bands were obtained. In order to analyze the suitability of SSR markers for a genetic profile evaluation, the performance of SSR markers used was measured by estimating the polymorphic information content (PIC), marker index (MI) and resolving power (Rp). The PIC which measures the informativeness of SSR markers was calculated for each SSR marker according to the formula:

$$PIC = 1 - n\Sigma\, pi^2$$

Where,

n = Total number of alleles detected for a locus of a marker.

pi = Frequency of the ith allele (Smith *et al*., 1997).

Marker index (MI) is a statistical parameter used to estimate the total utility of the maker system. The MI was determined as the product of PIC value and EMR (Prevost and Wilkinson, 1999). The effective multiplex ratio (EMR) of a primer is calculated as the total number of polymorphic loci multiplied by the proportion of polymorphic loci per their total number. Resolving power (Rp) is a parameter used to characterize the ability of the primer/marker combination to detect the differences between a large number of genotypes and was calculated as,

$$Rp = \Sigma I_b$$

Where,

I_b = Amplicon informativeness and calculated by the formula.

$$I_b = 1 - [2 \times (0.5 - p)]$$

Where,

p = Proportion of genotypes containing the band.

The binary data matrix was converted into a genetic similarity coefficient between pairs of accessions using the Jaccard's similarity coefficient by NTSYS-pc (Numerical taxonomy system, version 2.2 (Rohlf, 2005). The SIMQUAL Programme was used to calculate the Jaccard's similarity coefficient, a common estimator of genetic identity and was calculated as follows (Jaccard, 1908).

$$\text{Jaccard's coefficient} = \frac{N_{AB}}{N_{AB} + N_A + N_B}$$

Where,

N_{AB} = Number of bands shared by samples.

N_A and N_B = Amplified fragments in sample A and B respectively.

The matrix of Jaccard's similarity coefficients was subjected to the unweighted pair group method with arithmetic averages (UPGMA) to generate a dendrogram for cluster analysis which depicting the similarity and diversity among genotypes (Sokal and Michener, 1958; Sneath and Sokal, 1973).

RESULTS AND DISCUSSION

Characterization of genetic variation

Seventeen out of the twenty-five SSR primers used in the present investigation showed amplification in all the genotypes. A total of 102 alleles were detected in the 22 greengram genotypes (20 selected superior mutant cultures and their parents) using these seventeen SSR markers. The total number of alleles observed for each primer scored separately and percentage polymorphism calculated subsequently (Table 2). All the amplified bands were polymorphic in nature indicated that all SSR markers exhibited polymorphism. The polymorphism is due to the mutations in both the SSR regions and the flanking regions contributing to variation in allele size and number among different mutants. Gupta *et al*. (2013) had studied 30 SSR markers in mungbean genotypes, out of which 19 (63%) markers exhibited polymorphism. In this study, the number of alleles per locus varied from four to nine (Fig 1), with a mean of six alleles per locus. Sangiri *et al*. (2007) reported a higher average alleles per locus (16.3 alleles) for mungbean whereas Singh *et al*. (2014) and Kaur *et al*. (2018) observed less number of average alleles per locus *i.e*. 3.62 and 1.50, respectively. Reddy *et al*. (2008) reported that accessions with the most distinct DNA profiles are likely to contain the greatest number of novel alleles.

Polymorphism information of genetic diversity

The PIC value of a marker depends on the number of alleles and their relative frequencies. In this investigation, the PIC value which is a measure of the allelic diversity ranged from 0.450 to 0.804 with a mean value of 0.6335 and the PIC values of 14 SSR primers were higher than the mean value. The MI values ranged between 2.250 and 7.029. The maximum MI was observed for the primer SSRP 36 and the minimum MI (2.250) was obtained with SSRP 48 primer. The EMR of different primer varied from 4 to 9 with a mean value of 6. The SSR markers exhibited high EMR and MI and were quite effective in resolving the polymorphism content in studied

Table 1: Details of the greengram genotypes used in this study.

Sr no.	Mutants	Mutagen	Parent	Significant changes in phenotypic characters in comparison to their parent varieties
1	SG1-1	Gamma-rays (20 kR)	Sujata	Early maturity, increase in plant height, pods/pl., pod length and 100-seed weight
2.	SG1-2	Gamma-rays (20 kR)	Sujata	Early maturity, increase in plant height, pod length and 100-seed weight
3.	SG3-3	Gamma-rays (60 kR)	Sujata	increase in plant height, pod length and seeds/pod
4.	SE1-2	EMS (0.2%)	Sujata	Increase in plant height, pods/plant, pod length, seeds/pod and 100-seed weight
5.	SE2-2	EMS (0.4%)	Sujata	Late flowering and increase in 100-seed weight
6.	SE2-3	EMS (0.4%)	Sujata	Late flowering, increase in plant height, pod length and 100-seed weight
7.	SE3-2	EMS (0.6%)	Sujata	Early maturity, increase in plant height, pods/pl., pod length, seeds/pod and 100-seed weight
8.	SN1-2	NG (0.005%)	Sujata	Late flowering, Increase in pods/plant and 100-seed weight
9.	SN3-3	NG (0.015%)	Sujata	Late flowering, Increase in pods/plant, pod length and 100-seed weight
10.	SM1-3	MH (0.01%)	Sujata	Late flowering, increase in pod per plant and 100-seed weight
11.	OG1-1	Gamma-rays (20 kR)	OBGG-52	Increase in pod/plant, pod length and seeds/pod
12.	OG3-2	Gamma-rays (60 kR)	OBGG-52	Increase in pod length and seeds/pod
13.	OG3-3	Gamma-rays (60 kR)	OBGG-52	Increase in pod/plant, pod length and seeds/pod
14.	OE1-2	EMS (0.2%)	OBGG-52	Increase in plant height and seeds/pod
15.	OE2-3	EMS (0.4%)	OBGG-52	Increase in seeds/pod
16.	ON3-1	NG(0.015%)	OBGG-52	Increase in plant height, pods/plant and seeds/pod
17.	ON3-2	NG(0.015%)	OBGG-52	Late flowering, increase in pod/plant, pod length and seeds/pod
18.	ON3-3	NG(0.015%)	OBGG-52	Increase in seeds/pod
19.	OM1-3	MH (0.01%)	OBGG-52	Late flowering and Increase in seeds/pod
20.	OGN2-3	Gamma-rays (40 kR) + NG (0.01%)	OBGG-52	Shorter in plant height and increase in seeds/pod
21.	Sujata	-	L24-2 × Pusa Baisakhi	-
22.	OBGG	-	Mutant of K 851	-

Table 2: Details of SSR primers showed amplification in this study.

Primer no.		Primer sequences forward (F) and reverse (R)	Range of amplicons (in bp)	Total amplified bands	No. of polymorphic bands	PIC	MI	Rp
SSRP 01	F	CTTGCTTGCGAGGATGAC	150-600	7	7	0.595	4.165	2.182
	R	TCCAGTGCAGCAAGTTGA						
SSRP 03	F	GCAGCAACAACAGCAACA	100-500	7	7	0.676	4.732	2.273
	R	GCAGGTTTTGTGGCTCAG						
SSRP 11	F	CTTTGCCACACTCCTTGC	100-600	5	5	0.694	3.47	2.364
	R	AGCCAGGTGCAAGACCTTAG						
SSRP 13	F	AGATAGGGAGCTGGTGGTAGC	100-900	6	6	0.783	4.698	2.091
	R	AGTAAATCACCCGCACGATCG						
SSRP 15	F	GGAGAGGAAGGAACAGGG	100-500	7	7	0.775	5.425	2.182
	R	AGGGGAAATGGAGAGGACGAT						
SSRP 17	F	AGCGACGGCAACAACGAT	100-1500	7	7	0.802	5.614	2.182
	R	TTCCTTGCAACAAAAATACA						
SSRP 19	F	GAGGACCCAATAGAAGTGAAAAT	100-400	5	5	0.700	3.50	2.273
	R	TCGGCCAGCAACAGATGC						
SSRP 23	F	CGGGAATTAACGGAGTCACC	100-900	6	6	0.684	4.104	2.273
	R	CCCAGAGGCCGCTATTACAC						
SSRP 25	F	GCGGGTAGTGTATACAATTTG	100-350	5	5	0.804	4.02	1.728
	R	GTACTGTTCCATGGAAGATCT						
SSRP 36	F	CAAGGCATGGAAAGAAGTAAGAT	550-2000	9	9	0.781	7.029	2.455
	R	TCGAAGCAACAAATGGTCACAC						
SSRP 38	F	AGCCCTTCGTGCTAGGAAAT	200-400	5	5	0.769	3.845	2.000
	R	CCCTACCGGTTGGTTGGT						
SSRP 42	F	TGGTTGGTTGGTTCACAAGA	100-300	4	4	0.725	2.90	2.000
	R	CACGGGTTCTGTCTCCAATA						
SSRP 44	F	TCACAAAGGGAGGGAAGAGA	200-400	5	5	0.746	3.73	2.091
	R	CCCCAGGTTGGTTGGTTGGA						
SSRP 46	F	GATGAAGACCCCTTCACAGC	250-1000	9	9	0.630	5.67	2.636
	R	GTTCACCCTCGGTTGGTTGG						
SSRP 48	F	TGACGGAGAGACAGAGACAGAG	150-1250	5	5	0.450	2.25	2.000
	R	TGCTTCCTTTTGTCTGAGTTAGAA						
SSRP 50	F	GTAGCGCAGAGAGAGAGAGAG	200-1500	5	5	0.659	3.295	2.091
	R	CAAAACGGCTCATTCAGCTT						
SSRP 52	F	GAGAGCAACGTATGAAAAATG	100-300	5	5	0.756	3.78	2.000
	R	GTTCGTAGTTACATTGTCCC						
		Total		102	102	-	-	
		Mean		6	6	0.6335	4.249	2.165

genotypes and indicated their suitability for better analysis of genetic diversity in greengram. The estimates of Rp ranged from 1.728 to 2.636 with an average of 2.165 per primer. The highest Rp was recorded for the primer SSRP 46 (2.636) followed by SSRP 36 (2.455) and the lowest value was scored with the primer SSRP 25 (1.728).

Table 3: Grouping of mutant genotypes into different genetic clusters on the basis of polymorphic banding patterns.

Cluster no.	Number of mutants	Name of the mutant genotypes
I	2	SG1-1, SE1-2
II	1	SG1-2
III	2	SN1-2, SN3-3
IV	2	SE 3-2, SM 1-3
V	2	SG3-3, SE2-2
VI	1	SUJATA
VII	1	SE2-3
VIII	1	OG1-1
IX	1	OM1-3
X	2	OGN2-3, OBGG-52
XI	5	OG3-2, OG3-3, OE2-3, ON3-1, ON3-3
XII	1	OE1-2
XIII	1	ON3-2

Genetic relationship among genotypes

The dendrogram (Fig 2) constructed based on Jaccard's similarity coefficient and UPGMA with the SSR data from 102 polymorphic loci clearly formed two major groups at a 70% cut level of similarity, representing the parents Sujata and OBGG-52 along with their mutants. These two major groups divide into 13 clusters (Seven clusters belong to the Sujata group and the other six clusters belong to OBGG 52 group) at a 38% cut level of similarity. The Jaccard's similarity coefficient ranged from 0.07 to 0.70 with an average value of 0.38. Maximum five of mutant genotypes (OG3-2, OG3-3, OE2-3, ON3-1, ON3-3) included in cluster XI. The remaining 15 mutant cultures and their parent are distributed among the remaining 12 clusters (Table 3). Clusters I, III, IV, V and X individually consisted of two accessions and all other clusters consisted of single accessions. Seven mutant genotypes of Sujata (SG1-1, SG1-2, SE1-2, SE3-2, SN1-2, SN3-3, SM1-3) and eight mutant genotypes of OBGG-52 (OG1-1, OG3-2, OG3-3, OE1-2, OE2-3, ON3-1, ON3-2, ON3-3) were recorded maximum genetically diverse from their respective parent. Similar clustering through UPGMA

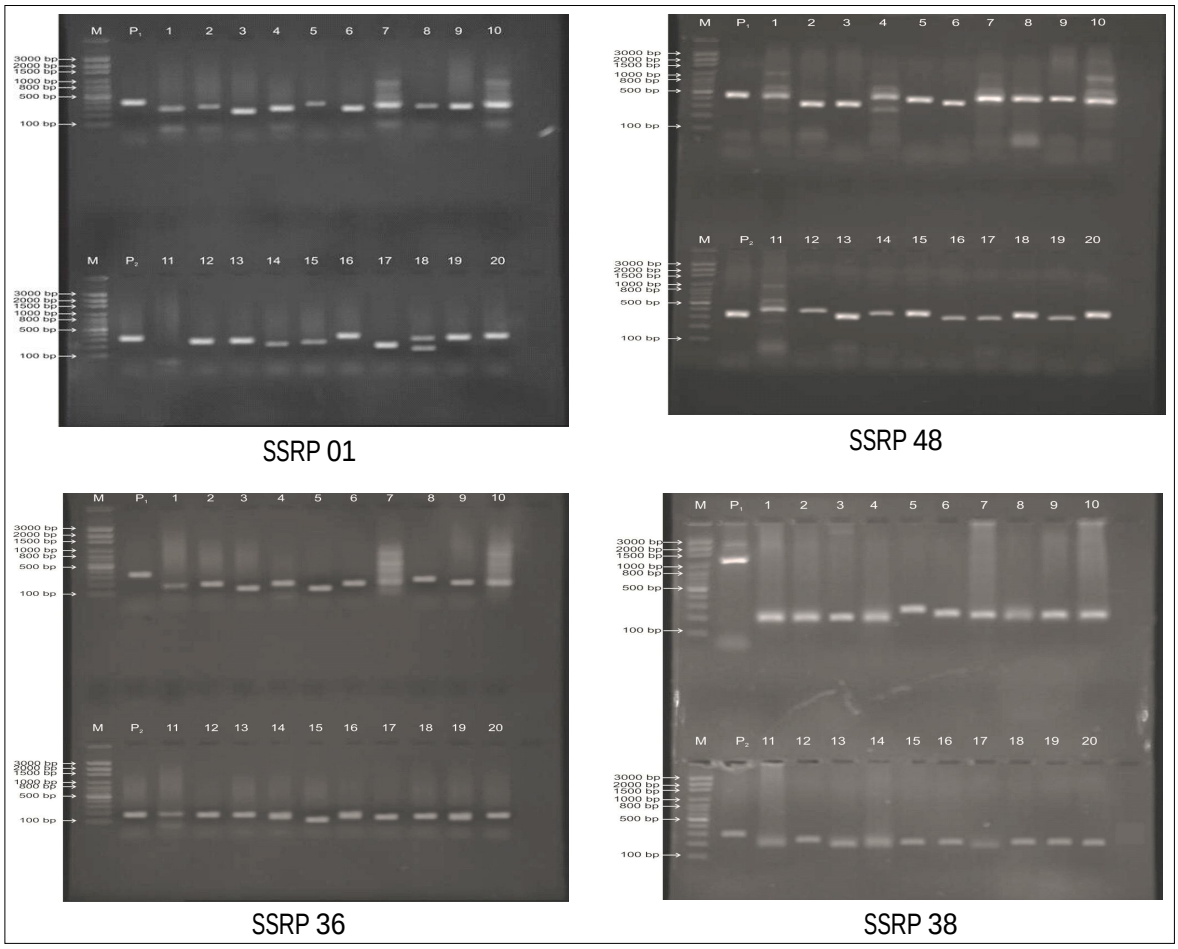

Fig 1: DNA polymorphism among greengram genotypes revealed by SSR primers.

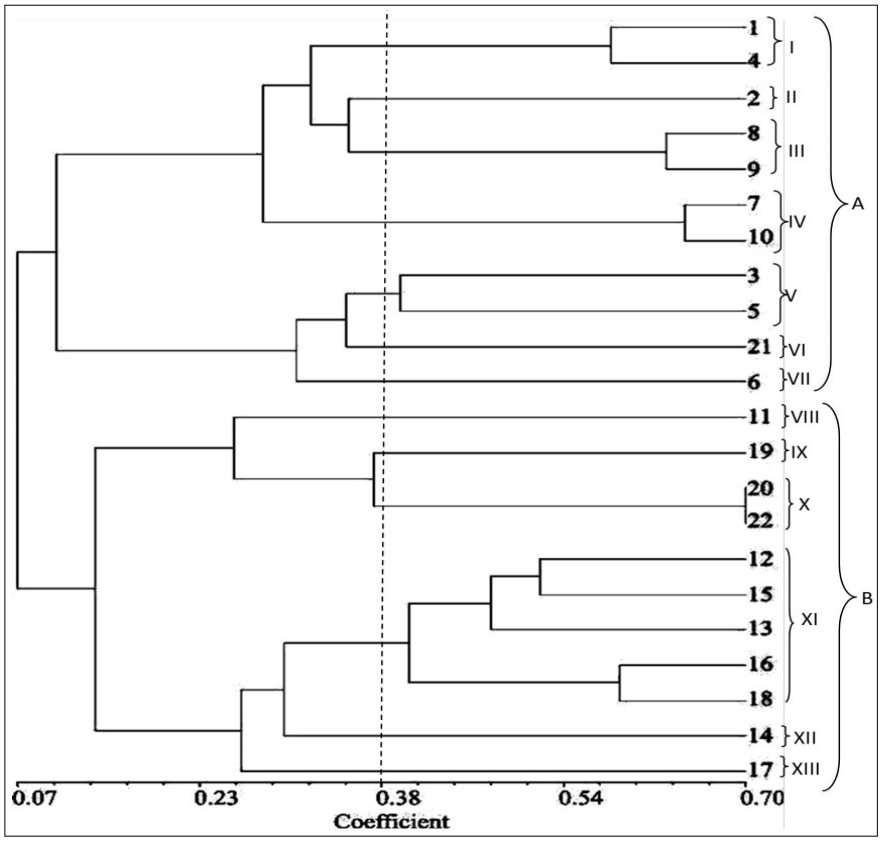

Fig 2: Dendrogram obtained by UPGMA analysis based on SSR marker data of 22 greengram genotypes.

analysis based on 19 polymorphic SSR markers grouped the 29 mungbean genotypes into three main clusters with the similarity coefficient ranged from 0.31 to 1.00 (Gupta *et al*., 2013). Reddy *et al*. (2008) reported those 30 greengram genotypes were grouped into five clusters with Jaccard's similarity coefficients ranged from 0.64 to 0.90. Similar polymorphism results have been reported by Singh *et al*. (2012) where they estimated the genetic diversity of the induced variation among 30 mutant lines and parent genotypes. Sherawat *et al*. (2014) studied thirty-eight novel microsatellite markers (SSRs) specific to candidate genes for detection of genetic variations in twenty-two greengram genotypes and reported the maximum similarity value of 0.56.

CONCLUSION

The present study revealed that SSR markers are very effective in detecting the genetic variation in greengram and could be well utilized to analyze the mutants at the DNA level. Each polymorphic SSR marker detected four to nine alleles with an average of six alleles per locus. On the basis of genetic diversity study through SSR markers, it could be concluded that genotypes are falling in same group existing possibility of many traits to be similar whereas the genotypes falling extremely apart from the cluster represents the possibility of a considerable degree of genetic variation and discovering new traits in them which could be further useful in the selection of the suitable genotypes and SSR markers for future breeding programmes to improve yield and other desirable characters in greengram. The study also suggests that the genotypes with high genetic diversity can be exploited as parental lines in recombination breeding programmes to develop promising transgressive segregants.

Conflict of interest: None.

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