Molecular Characterization and Diversity Analysis of Green Gram [*Vigna radiate* (L.) Wilczek] by Using SSR Markers

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Original Research Article	

ABSTRACT

Molecular characterization was done for Green gram genotypes using SSR markers. The 14 green gram genotypes were clustered using ward method and squared Euclidean distance using nine SSR markers. As per dissimilarity coefficient, genotypes were grouped into three clusters namely Cluster I which consisted of 6 genotypes and divided into two sub Clusters IA consist of IPM2-3, IPM-99-125, PUSA-0672, DGGV-2 and sub cluster IB consists of MH-2-5, HUM-16 Cluster II and Cluster III, Cluster II consisted of 4 genotypes that was further divided into three sub clusters IIA consisted of MH-3-18, PUSA VSHAL and sub cluster IIB consisted of one genotype namely PANT MUNG-5, sub cluster IIC had PUSA-9531. Cluster III consisted of 4 genotypes divided into two sub clusters IIIA consists of SML-668, SML-832 and sub cluster IIIB consisted of IPMD-604-1-7, OBGG-58. Polymorphic information content values (PIC) ranged from 0.000 to 0.704 per locus with an Average value of 0.519. Among 9 markers SSR VR91 generated highest percent of PIC value.

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Based on the polymorphic band results PCA was performed for 9 SSR markers for 14 Green gram genotypes among all the factors PC_1 TO PC_9, the PC_1 (25.872) accounted for maximum proportion of variability in the set of all variables and PC_9 (0.372) accounted for progressively lesser amount of variability. It can be concluded from the above results that the highest Eigen value for PC_1 (2.328) and the least is PC_9 with (0.033) were registered.

Keywords: Molecular characterization; green gram; PIC; SSR markers.

1. INTRODUCTION

Greengram [*Vigna radiata* (L.) Wilczek], also known as mungbean, is an ancient pulse crop widely cultivated in India. It is a diploid species with the chromosomal number 2n=2x=22, a member of the subfamily Papilionaceae of the Leguminosae family, and its botanical name is *Vigna radiata* (L.) Wilczek. Mungbean is a native of South Asia (India), and its likely ancestor is *Vigna radiata var. sublobata*. Genetic diversity assessment in available cultivars has important implications in understanding the progress made in any breeding programme. Morphological markers are routinely used for estimating the genetic diversity, but recently molecular marker techniques have become powerful tools to analyze genetic relationships [1,2].

Molecular characterization is also useful in understanding the phylogenetic relationship between plant species to reveal the genetic diversity within a given taxonomic group. Evaluation of genetic diversity would promote the efficient use of genetic variations in conservation [3,4]. The determination of purity of genotypes and utilization of germplasm in crop improvement are also possible with molecular markers [5,6]. DNA Fingerprinting is one of the important applications of molecular markers which allows identifying an individual from others by using variations at DNA level.

Microsatellites or simple sequence repeats (SSRs) are clusters of short tandem repeated nucleotide bases distributed throughout the genome and are valuable for genetic mapping, genotyping and marker-assisted selection in breeding due to their co-dominant nature, higher

allelic variation and even distribution in the genome [7,8]. Major features that made SSRs very popular are their abundant distribution in the genomes examined to date and their hyper variable nature [9]. In greengram, the variation observed for these markers is very less indicating the necessity to use more and more number of markers to get the clear picture of diversity and use it for fingerprinting of the genotypes.

2. MATERIALS AND METHODS

Molecular Diversity in Green gram genotypes using SSR markers was conducted during *Rabi* 2021-2022 at the field experimentation centre and Seed Testing Laboratory of the Department of Genetics and Plant Breeding, Sam Higginbottom Institute of Agriculture, Technology and Sciences Deemed to be University, Allahabad followed by molecular work at Division of Plant Biotechnology, Indian Institute of Pulses Research (IIPR), Kanpur. The experimental material consisted of 14 diverse lines of green gram (PUSA VISHAAL, IIPM-99-125, SML-832, DGGV-2, IPMD-604-1-7, PANT MUNGS-5, SML-668, PUSA 9531, OBGG-58, IPM-2-3, MH-3-18, PUSA 0672, HUM-16, MH-2-5).

2.1 Genomic DNA Extraction and Quantification

DNA was extracted from leaf tissue collected at 21 days after sowing from all the entries using the method described by [10]. The extraction buffer was prepared by using the chemicals (Table 1) and stock solutions as per the procedure.

Table 1. Chemicals required for preparation of extraction buffer

S. No.	Components	Stock Concentration	Working Concentration	Volume taken for 500 ml
1	Tris HCL	1M	200 mm	100 ml
2	EDTA	0.5M	20 mm	20 ml
3	NaCl	5M	1.4 mm	140 ml
4	CTAB	2%	2 g	10 g

2.2 SSR-PCR Amplification

The polymerase chain reaction (PCR) was carried out using a programmable thermocycler. 96 well PCR plates were used and 2.5 µl of 20 ng template DNA was pipetted into each of the PCR well after proper labelling and kept the PCR plate at 4^0C.

The PCR products were analyzed by electrophoresis using a 3% high resolution agarose gel using a horizontal gel Electrophoresis Unit. The DNA fragments were then visualized under UV-transilluminator and documented using gel documentation system.

Total of nine markers (Table 2) were used for characterization of 14 green gram genotypes and the amplification products were viewed under UV light and photographs were saved for the experimental evaluation. The amplification products were scored separately for each primer.

The bands were scored for the presence or absence by binary coding i.e., assigning a value of 1 for presence and 0 for absence in a lane.

2.3 Data Analysis

The amplification data obtained from SSR primers were used for a combined similarity matrix and to generate a combined dendrogram. Dendrogram of all test genotypes of greengram were constructed on the basis of presence or absence of SSR bands using the UPGMA (unweighted pair-group method with arithmetic mean) cluster analysis. Principal component analysis (PCA) was also used for non hierarchical relationships among the genotypes. Eigenvalues and eigenvectors were calculated by the Eigen program using a correlation matrix as input (calculated using standardized molecular data), and 2D and 3D plots were used to generate the two-dimensional PCA plot from XLstat version 2021.

Chart 1. Specification for polymerase chain reaction

Cycle	Denaturation		Annealing		Polymerization	
	Temp.	Time	Temp.	Time	Temp.	Time
First cycle	94˚C	5 min	-	-	-	-
39 cycles	94˚C	1 min	46-51˚C	2 min	72˚C	2 min
Last cycle	-	-	-	-	72˚C	10 min

Annealing temperature for each marker was standardized using gradient

Table 2. Nine SSR Markers with sequence and melting temperature (Tm) and annealing temperature (AT)

No	Primers	Sequences	Tm	Length(bases)
1	VR 80	R=51AATGGTCCCTTTACCCCTTTT	50.5℃	21
		F=5ˈTGTGAGAGTGGAAGAGCAACTT	50.5℃	22
2	VR 48	R=5ˈAATAGGGCCCATAACATGTCC	52.4℃	21
		F=5ˈAGGTGAGTGAAAATTGGAATAGG	51.7℃	23
3	VR 91	R=5ˈTGGAGATGCAGGACTAAGAAGAG	55.3℃	23
		F=5ˈACATATGTATCTGTCTGTGTGCCTA	54.4℃	25
4	VR 86	R=5ˈATCGGTATATGTTGCCAATCAG	51.1℃	22
		F=5ˈCTATACTGCAATGAAGTGGATCTC	54.0℃	24
5	CEDG168	R=5ˈCATTACATTCCAGACCTGC	58.01℃	22
		F=5ˈCTGCTTGGTGTTGAGCTTC	57.8℃	25
6	CEDG030	R=5ˈTCGGCAGATAGAGGCTCACG	58.8℃	23
		F=5ˈTGAGGGAATGGGAGAGAGGC	58.6℃	22
7	CEDG050	R=5ˈGAGATTATCTTCTGGGCAGCAAGG	61.97℃	22
		F=5ˈTCCCACTTGTCCATTACCTCCAC	61.95℃	25
8	CEDG097	R=5ˈTGCCAAAGAGCCGTTAGTAGAA	58.21℃	23
		F=5ˈGTAAGCCGCATCCATAATTCCA	58.21℃	21
9	CEDG156	R=5ˈCTTAGTGTTGGGTTGGTCGTAAGG	61.97℃	21
		F=5ˈCGCGTATTGGTGACTAGGTATG	60.07℃	25

2.4 Polymorphic Information Content

Polymorphic information content (PIC) that provides an estimate of the discriminatory power of a locus or loci, by taking into account, was calculated using the formula given by Smith et al., [11].

$$PIC = 1 - \sum_{f=1}^{n} P_{ij}^2$$

Where P$_{ij}$ is the frequency of the jth allele for the ith marker, and is summed over n alleles. The calculation was based on the number of alleles per locus.

3. RESULTS AND DISCUSSION

3.1 SSR Analysis

The present study evaluated the genetic diversity of 14 green gram genotypes. The study of genetic diversity in any breeding population is essential as it constitutes the backbone of any breeding and crop improvement program. It helps in the development of crop that is suitable and adaptable to rapid climate change through the introduction of foreign genes. Thus, genetic diversity is needed for developing ideal and desired crop varieties for present and future needs. In this study, 30 SSR markers were screened, out of which 9 SSR markers were found to be polymorphic and suitable for diversity analysis. The use of SSR for green gram diversity study is very crucial as it provides accurate and unbiased assessment and reveals in depth information on the genetic divergence of the material. SSR markers have been widely recognized for its codominant inheritance

pattern, high informative power, and transferability among the species, hence, its superiority as are the markers of choice for plant improvement program.

Based on the 9 SSR markers screened, 8 markers displayed clear and repeatable polymorphic bands, were selected for further analysis as shown in Figs. 1 and 2. A total of 20 alleles were recorded, and the number of alleles per locus ranged from 2 in Vr80, Vr86, CEDG 168, CEDG 030, CEDG 50 and CEDG 156 to 5 in Vr91 with an average of 2.22. The expected heterozygosity differed among the markers and ranged from 0.459 (Vr80) to 0.745 (Vr91) with an average of 0.60.

The polymorphic information content (PIC) was used as a measure to know the polymorphism of a marker locus and genetic diversity evolution. The PIC values were varied and are considered as informative when the values are more than 0.50 and PIC values of less than 0.25 are considered as low level of diversity. PIC values are moderately informative if values are 0.25 to 0.50. PIC exhibited significant difference, from 0.354 (Vr80) to 0.704 (Vr91). Similar results were also reported by Kushwaha et al. (2013), Andeden et al. (2013), Karthikeyan et al. [12] and Suvan et al. [13] for PIC values ranging from medium to high, while Gopal Krishna et al. (2010) and Kumar et al. [14] reported low PIC values. Hence, these SSR markers with high PIC values can be used for diversity studies, gene mapping and to know germplasm evolution.

Shannon's information index (I) was employed to assess the diversity among species in the present study. Shannon [15] yielded a high diversity result of 1.470 for VR91, while the

Table 3. Information generated by using 8 SSR markers in green gram genotypes

Markers	Alleles count	No. of effective alleles	Observed heterozygosity	Expected heterozygosity	PIC	I
Vr80	2.00	1.85	0.00	0.459	0.354	0.652
Vr48	3.00	2.09	0.00	0.520	0.464	0.892
Vr91	5.00	3.92	0.00	0.745	0.704	1.470
Vr86	2.00	1.96	0.00	0.490	0.370	0.683
CEDG 168	2.00	2.00	1.00	0.500	0.375	0.693
CEDG 030	2.00	2.00	1.00	0.500	0.375	0.693
CEDG 50	2.00	2.00	1.00	0.500	0.375	0.693
CEDG 097	0.00	0.00	0.00	0.000	0.000	0.000
CEDG 156	2.00	2.00	1.00	0.500	0.375	0.693
Total	20.00	15.81	4.00	4.214	3.392	5.776
Mean	2.22	1.98	0.44	0.468	0.519	0.641

PIC: Polymorphism information content; I: Shannon's diversity index

lowest diversity score of 0.000 was observed for CEDG097. On average, Shannon's index recorded a value of 0.641 across the 9 SSR polymorphic markers, as presented in Table 3.

Results from the present investigation revealed remarkably abundant genetic variation among the 14 greengram genotypes. The number of alleles ranged from 2 to 5. The number of alleles indicates the richness of the population. Since SSR are short tandem repeats, generally allele numbers of 2 to 5 alleles per locus are considered good as seen in this study. The PIC value ranged from 0.354 to 0.704 with an average of 0.519. The richness of information a marker can give, otherwise known as PIC reported in this study, was very interesting.

High PIC of 0.354 to 0.704 as seen in this study revealed that the markers have the required properties to be used in breeding program. Genetic diversity indices such as expected heterozygosity as well as Shannon's index among the markers were very high, thus reflecting the heterozygous nature of the population.

The average number of alleles per locus (Na) varied from 2.00 to 5.00. The effective number of alleles per locus (Ne) was small compared to the number of alleles per locus and it ranged from 1.849 to 3.920 with the mean number of 1.979. Shannon's information index in the present study was another indication of the presence of high genetic diversity in the green gram genotypes under consideration.
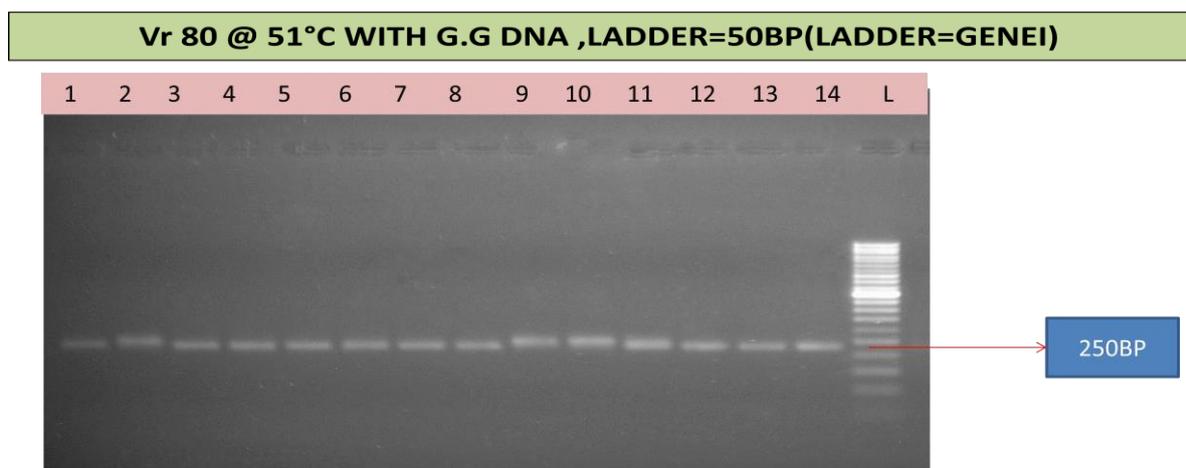

Fig. 1. Image of DNA banding pattern generated by agarose gel electrophoresis of SSR VR 80 marker for 14 green gram genotypes
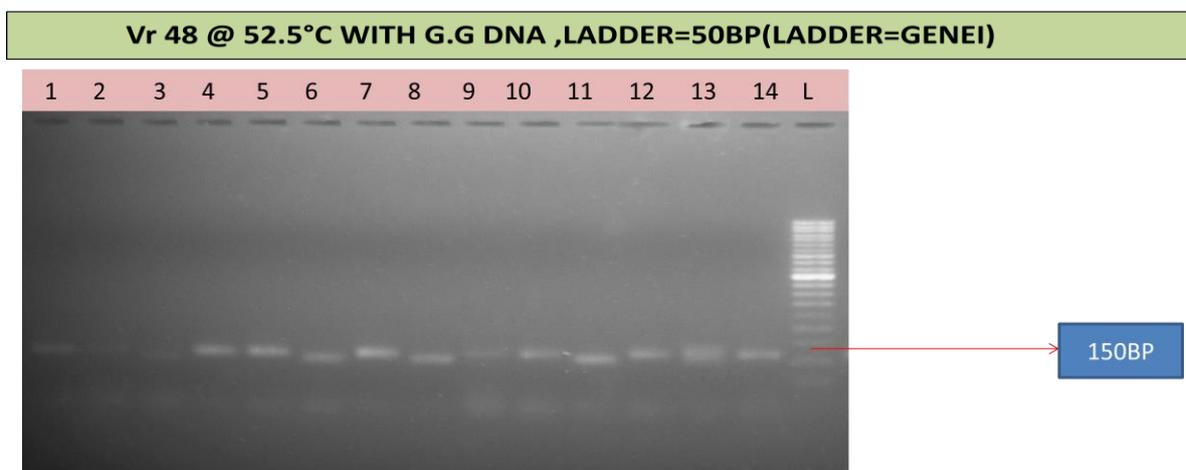

Fig. 2. Image of DNA banding pattern generated by agarose gel electrophoresis of SSR VR 48 marker for 14 green gram genotypes

3.2 Principal Component Analysis

Principal coordinate analysis (PCoA/ PCA) for morphological (visually assessed and measurable characters) and molecular markers were carried out to corroborate and validate the pattern of cluster analysis based on UPGMA method. PCA is utilized to drive a 2- dimensional and 3- dimensional scatter plot of individuals, such that geometrical distances among individuals in the plot reflect the genetic distances among them with minimal distortion.

In the present study, selected 14 genotypes were characterized according to 9 groups by PCA analysis. Total variation was divided in to 9 principal components. The initial four principal components (PCs), possessing eigenvalues exceeding 1 and collectively explaining 75.458% of the total variation, were chosen. These attributes were selected because they contribute more significantly to the overall variation than the remaining ones, as depicted in Fig. 3.

Eigenvalues, variability %, and cumulative contribution of each component to variation along with the contribution of each character to respective PCs are presented in Table 4. PC1 explained 25.872% of the total variability and PC2 with 19.644% contribution was highest. Factor loading of principal components and factor scores principal components of 14

genotypes of green gram genotypes are presented in Tables 5 and 6 respectively.

3.3 Biplot Analysis

In order to assess the diversity among the selected genotypes, they were plotted on a bi plot using the first two PCs, which possessed Eigen values exceeding 1 and contributed to 45.51% of the overall variability. Genotypes that are closely located on biplot, perceived as alike when rated on given attributes. More the distance between the point of origin and genotype, more diverse will be the genotypes from others. Regarding the first two PCs, genotypes were differentiated into four diverse groups (Fig. 4). On biplot, three genotypes i.e. PUSA VISHAAL, IIPM-99-125 and IPM-2-3 clogged far away from the origin and were considered as diverse from the others (Fig. 4). Remaining genotypes were clustered into three groups. The genotypes, namely DGGV-2, PUSA 0672, MH-2-5, and IPMD-604-1-7, clustered closely together and near the origin, indicating that these genotypes exhibit lower diversity and have limited breeding potential. Genotypes i.e. SML-832, SML-668 and OBGG-58 formed the third group while genotypes PANT MUNGS-5, PUSA 9531, MH-3-18 and HUM-16 were differentiated from rest of the genotypes and clustered in fourth group.

Table 4. Eigen values, %variance and cumulative eigen values of promising lines of greengram

Observations	Eigenvalue	Variability (%)	Cumulative %
P1	2.328	25.872	25.872
P2	1.768	19.644	45.516
P3	1.587	17.632	63.148
P4	1.108	12.310	75.458
P5	0.873	9.699	85.157
P6	0.716	7.954	93.111
P7	0.453	5.030	98.141
P8	0.134	1.487	99.628
P9	0.033	0.372	100.000

Table 5. Factor loading of principal components in Greengram

Observations	P1	P2	P3	P4	P5	P6	P7	P8	P9
Vr80	0.671	0.243	-0.199	0.095	0.294	-0.530	0.268	-0.033	0.052
Vr48	0.198	0.889	0.171	-0.193	-0.046	0.230	-0.192	0.018	0.104
Vr91	-0.346	-0.114	0.647	-0.382	-0.435	-0.209	0.229	-0.124	0.035
Vr86	0.594	-0.029	-0.267	-0.506	-0.012	0.433	0.360	0.013	-0.027
CEDG 168	0.690	-0.495	0.175	0.349	-0.011	0.251	-0.116	-0.218	0.042
CEDG 030	0.280	0.357	0.765	-0.121	0.421	-0.015	-0.064	-0.053	-0.097
CEDG 50	0.586	-0.570	0.490	-0.121	-0.045	-0.078	-0.100	0.241	0.036
CEDG 097	-0.644	-0.184	0.314	0.231	0.499	0.263	0.274	0.041	0.066
CEDG 156	0.231	0.416	0.271	0.674	-0.408	0.110	0.244	0.082	-0.034

Table 6. Factor scores principal components of 14 genotypes of greengram genotypes

| Observations	P1	P2	P3	P4	P5	P6	P7	P8	P9
PUSA VISHAAL	3.163	0.748	-1.418	-0.872	-1.853	-0.857	-0.694	-0.107	-0.058
IIPM-99-125	2.296	0.815	-0.057	-0.04	1.013	0.329	0.174	0.312	0.085
SML-832	-2.101	0.861	-1.479	0.484	-0.591	0.156	-0.246	0.124	0.474
DGGV-2	0.032	-0.061	1.196	0.526	0.442	0.436	-1.126	0.14	-0.041
IPMD-604-1-7	-0.176	0.462	-1.443	0.247	0.103	1.875	0.204	-0.774	-0.041
PANT MUNGS-5	-0.033	-2.38	-0.938	0.154	-0.791	0.801	0.978	0.357	-0.234
SML-668	-2.592	1.565	-0.913	-0.178	-0.314	-0.33	0.006	0.645	-0.253
PUSA 9531	-0.963	-2.736	-0.387	-0.315	0.567	-0.523	-0.944	-0.317	-0.046
OBGG-58	-0.788	1.904	-0.066	0.25	1.066	-1.282	0.461	-0.552	-0.222
IPM-2-3	1.924	0.4	0.129	0.126	1.301	0.361	0.629	0.304	0.089
MH-3-18	0.269	-1.703	-0.269	1.206	0.314	-1.558	0.642	-0.038	0.17
PUSA 0672	0.064	0.004	1.24	0.66	0.352	0.463	-1.05	0.186	-0.079
HUM-16	-0.889	-0.313	1.395	-3.307	0.147	0.032	0.385	-0.059	0.124
MH-2-5	-0.206	0.434	3.01	1.058	-1.757	0.098	0.58	-0.222	0.03
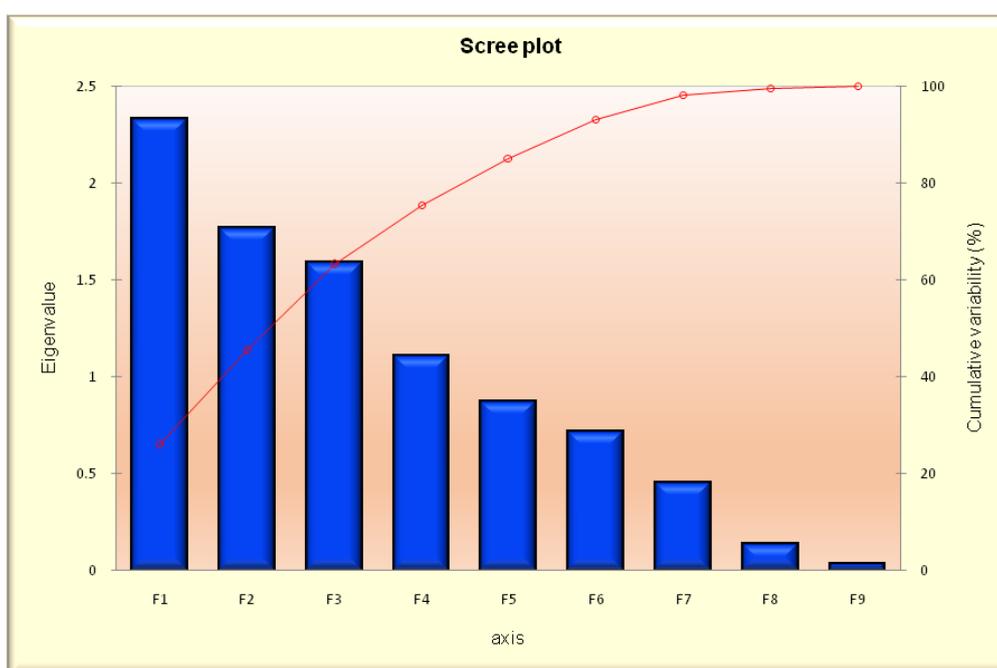
Fig. 3. Screen plot showing PCA factors eigen value and cumulative variability

3.4 Cluster Analysis

The 14 green gram genotypes were clustered using the Ward method with squared Euclidean distance as the metric, based on data from 9 SSR markers. Dendrogram showing genetic relationship among 14 greengram genotypes based on Euclidean's coefficient and UPGMA analysis using molecular characterization of the aforesaid loci and designated into three major clusters (Fig. 5). As per dissimilarity coefficient, genotypes were grouped into three clusters namely Cluster I and Cluster II and Cluster III.

Cluster I comprises 6 genotypes, which are further divided into two sub clusters. Sub cluster IA includes IPM2-3, IPM-99-125, PUSA-0672, and DGGV-2, while sub cluster IB consists of MH-2-5 and HUM-16.Cluster II consists of 4 genotypes divided into three sub clusters IIA consists of MH-3-18, PUSA VSHAL and sub cluster IIB consists of one genotype namely PANT MUNG-5, sub cluster IIC has PUSA-9531. Cluster III consist of 4 genotypes divided into two sub clusters IIIA consists of SML-668, SML-832 and sub cluster IIIB consist of IPMD-604-1-7 and OBGG-58. These results were in agreement with the reports by Sharma and Sirohi [16], Kanavi et al. [17] and Tabasum et al. [18].
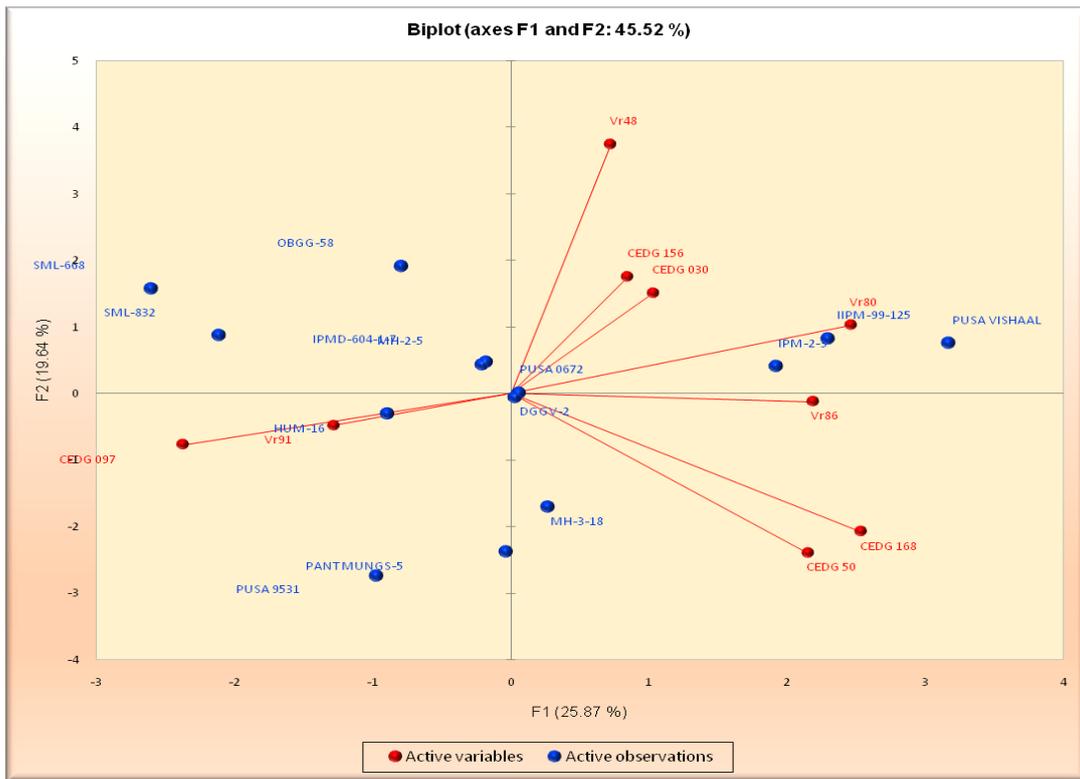
Fig. 4. Distribution of 20 genotypes on the bi-plot axes in PC 1 and PC2 of principal component analysis
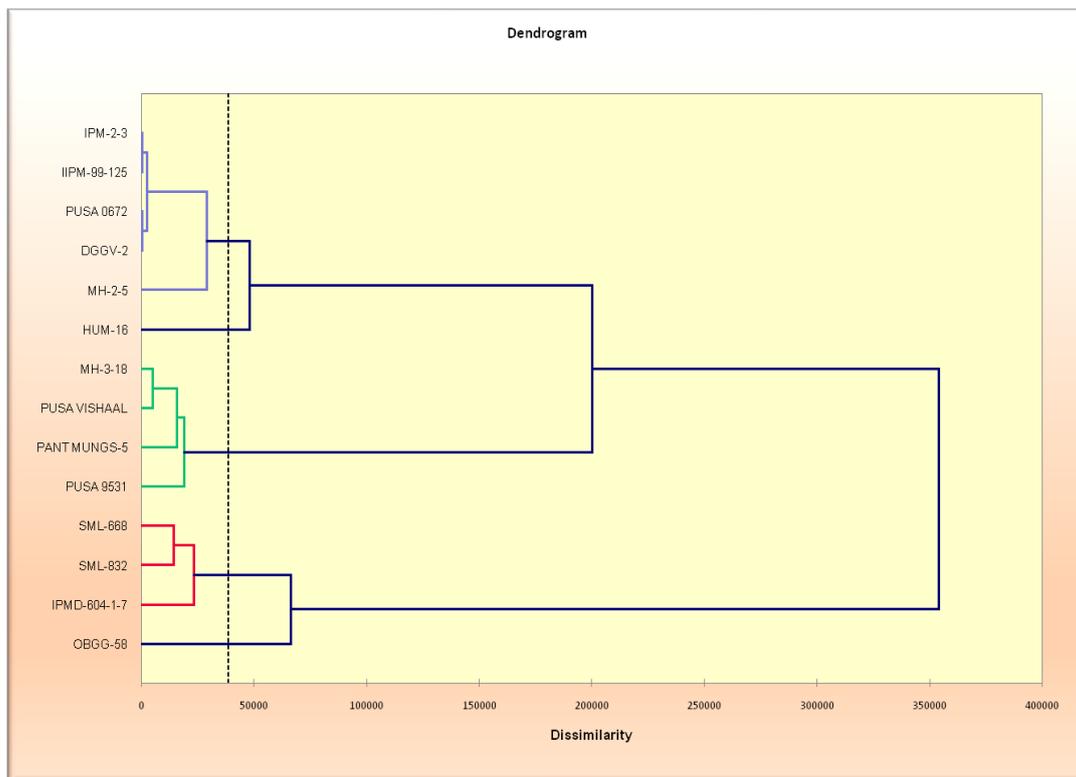

Fig. 5. Cluster analysis using ward method and squared euclidean distance for 14 green gram genotypes

4. CONCLUSION

No variation was observed between the clusters generated through Euclidean dissimilarity and PCA analysis for measurable characters. The major clusters generated in both the cases were same. PCA revealed a more distinct and well-defined structure compared to the UPGMA dendrogram. This enhanced presentation was achieved by utilizing two and three dimensions in PCA, as opposed to only one dimension in the dendrogram. It allowed for a clearer differentiation in the relatedness of certain genotypes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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