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ASSESSMENT OF MOLECULAR DIVERSITY OF SOYBEAN [*Glycine max* (L.) Merrill]" VARIETIES USING SSR MARKERS FOR EFFECTIVE SELECTION AND IMPROVEMENT

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ABSTRACT
The present experiment was taken up to study molecular marker diversity analysis of Soybean varieties using sixteen different SSR primers for effective selection and improvement of crop plant. The assessment of SSR diversity of twenty soybean genotypes revealed that the Jaccard's similarity coefficient estimates between pair of different varieties varied from 0.59 [between Kalitur and JS 335, Ankur and Bhatt] to 0.92 [between PK 472 and Ankur]. The average number of alleles per primer was 4.125, while percentage of all bands that showed polymorphism was 100%. The UPGMA ordered the population of twelve varieties into two main groups namely Group A and Group B and five clusters. Based on dendrogram PS 1347 and Ankur were found to be most distant.

KEYWORDS: Primer, SSR marker, molecular assessment, diversity, alleles.

INTRODUCTION
Soybean [*Glycine max* (L.) Merrill] is one of the most important oil seed crops throughout the world. Its oil is the largest component of the world's edible oils. Soybean seed contains 20% oil and 40% protein. Because of its multiple uses, this crop is aptly called as "Golden Bean" or "Miracle crop" of the 20th century. Several varieties have been released till date in India from different soybean breeding centers for growing under different agro-climatic conditions by different breeding methods such as: introduction, selection, mutation and hybridization of elite cultivars and germplasm through systemic breeding and evaluation programs (Chauhan *et al.*, 2015). The assessment of available genetic variability is of utmost importance in all the crop improvement programs. This is important for several reasons: the ability to distinguish reliably different genotypes for designing the breeding programs, population-genetic analysis, genetic engineering, and an estimation of the amount of variation within genotypes and between genotypes which is useful for predicting potential genetic gains in a breeding programme and in setting up appropriate cross-breeding strategies. Characters of economic importance are often controlled by multiple genes and are subjected to varying degree of environmental modifications and interactions hence, the differences between genotypes are not always absolute and reliable. Neutral molecular markers are considered to provide the best estimates of available genetic diversity since they are independent of the confounding effects of the environmental factors. Among different types of DNA markers being utilized for molecular characterization and genetic diversity analysis in plants, simple sequence repeats (SSR) markers are considered as molecular marker of choice due to their abundance, high polymorphism rate and high reproducibility (Song *et al.,* 1999; Cregan *et al.,* 1999). SSR markers have been widely used in the genetic diversity studies of the soybean germplasm collections worldwide and high levels of polymorphism at SSR loci have been reported for both the number of alleles per locus and the gene diversity (Maughan *et al.*, 1995; Abe *et al.*, 2003; Wang *et al.*, 2006, 2010; Fu *et al.*, 2007; Wang and Takahata 2007; Li *et al.*,2008; Singh *et al.*, 2010; Tantasawat *et al.*, 2011). Early studies have shown utilization of molecular markers for identification of genetically diverse genotypes to use in crosses in breeding programme (Maughan *et al.*, 1996; Thompson and Nelson 1998).
Therefore, understanding the genetic diversity of soybean germplasm is essential to broaden the genetic base and to further utilize it in breeding program. Knowledge on genetic diversity in soybean could help to predict combinations, which would produce the best offspring and facilitate to widen the genetic base of breeding material for selection. Genetic distances will further help in identifying genetically diverse genotypes, which then can be utilized in creating valuable selectable variation.

MATERIALS & METHOD
Plant materials
The experimental plant material in the present study consisted of twelve soybean varieties developed from different centers. The seeds were obtained from the department of Genetics & Plant Breeding, G. B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand. The details of varieties along with their

pedigree are given in **Table 1.**

Table 1: List of soybean varieties with their pedigree

S. No.	Varieties	Pedigree
1.	PS 1347	PS 1024 x PK 472
2.	PS 1225	PK 515 x PK 327
3.	JS 335	JS 78-77 x JS 71-5
4.	Bragg	Jackson x D 49-2491
5.	Bhatt	Indigenous strain
6.	PK 327	UPSM 82 x Semmes
7.	PK 1029	PK 262 x PK 317
8.	PS 1024	PK 308 x PK 317
9.	Kalitur	Indigenous strain
10.	PS 1092	PK 327 x PK 416
11.	PK 472	Hardee x Pb-1
12.	Ankur	A composite of 12 crosses

DNA Extraction

Isolation of high molecular weight plant genomic DNA is a prerequisite for molecular analysis. CTAB procedure was used for isolation of DNA (Doyle and Doyle, 1987). CTAB (Cetyl Trimethyl Ammonium bromide) is a cationic detergent which solubilizes membranes and forms a complex with DNA. After cell disruption and incubation with hot CTAB isolation buffer, proteins were extracted by chloroform: isoamyl alcohol. CTAB-DNA was precipitated with isopropanol. The DNA pellet resulting after centrifugation was washed, dried and re-dissolved. RNase treatment was given to remove RNA contamination.

Purification and quantification of genomic DNA

5µl RNase (10mg/ml) was added to 100 µl of dissolved DNA and incubated at 37°C for 1 hour. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently by inverting the tubes. The tubes were spun at 10,000 rpm for 5 min and the top layer of DNA was removed. To this, sodium acetate (1/10 vol, pH 5.2) and chilled absolute ethanol was added. The contents were mixed and kept at - 20°C for 30 min. Finally, the pellet was washed with 70 per cent ethanol, dried and dissolved in 100µl TE buffer. The quantification of genomic DNA was done by taking the absorbance on UV spectrophotometer. The optical density was measured at 260 nm and 280 nm. The concentration of the DNA in the sample is related to optical density by the following formula:

Concentration of DNA (µg/ml) = A_{260}nm x 50 x dilution factor/1000

The ratio of OD260/280 was an indication of the amount of RNA or protein contamination in the preparation. A value of 1.8 is optimum for best DNA preparation. A value of the ratio below 1.8 indicates the presence of protein in the preparation and a value above 1.8 indicates that the sample has RNA contamination.

PCR amplification

Sixteen SSRs primer pairs, distributed across the integrated linkage map of soybean were used (Cregan *et al.*, 1999). The details of SSR markers, their sequences and motifs are given in **(Table 2).** DNA was amplified by PCR using our previously standardized method (Sahu *et al.*, 2012) and amplification was carried out in a 15 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 25 mM MgCl2, 0.01% gelatine, 2.5mM each dNTPs, 20 SSR primers, 50ng/µl genomic DNA and 5U/µl Taq DNA polymerase (Bangalore Genei, Bangalore, India) **(Table 3).** Amplifications were performed in a Biored Master Cycler gradient. Amplified products were separated on 3 % agarose gel in 1X TAE buffer at 100V. The gel was stained with 0.5µg/ml ethidium bromide solution and visualized by illumination under UV light in gel doc system (Alpha Innotech, Alpha-Imager EC). The size of amplification products was determined by comparison to low range DNA Ruler plus marker.

Table 2: List of SSR primers

S. No.	Primer	Ends	Sequence	Tm °C
1.	Satt187 forward	Left end	GCGTTTTAATTTATGATATAACCAA	53.1
	Satt187 reverse	Right end	GCGTTTTATCTCTTTTTCCACAAC	57.6
2.	Satt228 forward	Left end	TCATAACGTAAGAGATGGTAAAACT	56.4
	Satt228 reverse	Right end	CATTATAAGAAAACGTGCTAAAGAG	56.4
3.	Satt577 forward	Left end	CAAGCTTAAGTCTTGGTCTTCTCT	59.3
	Satt577 reverse	Right end	GGCCTGACCCAAAACTAAGGGAAGTG	66.4
4.	Satt461 forward	Left end	AAATACAAGCTTTAATAAAGTGCAGA	55.3
	Satt461 reverse	Right end	CTTACGTTTCCATAGATTTCTCG	57.1
5.	Satt146 forward	Left end	AAGGGATCCCTCAACTGACTG	59.8
	Satt146 reverse	Right end	GTGGTGGTGGTGAAAACTATTAGAA	59.7
6.	Satt571 forward	Left end	GGGTAGGGGTGGAATATAAG	57.3

	Satt571 reverse	Right end	GCGGGATCCGCGGATGGTCAAAG	67.8
7.	Satt114 forward	Left end	GGGTTATCCTCCCCAATA	53.7
	Satt114 reverse	Right end	ATATGGGATGATAAGGTGAAA	52.0
8.	Satt268 forward	Left end	TCAGGGGTGGACCTATATAAAATA	57.6
	Satt268 reverse	Right end	CAGTGGTGGCAGATGTAGAA	57.3
9.	Satt586 forward	Left end	GCGGCCTCCAAACTCCAAGTAT	62.1
	Satt586 reverse	Right end	GCGCCCAAATGATTAATCACTCA	58.9
10.	Satt345 forward	Left end	CCCCTATTTCAAGAGAATAAGGAA	57.6
	Satt345 reverse	Right end	CCATGCTCTACATCTTCATCATC	58.9
11.	Satt300 forward	Left end	GCGCCCACACAACCTTTAATCTT	60.6
	Satt300 reverse	Right end	GCGGCGACTGTTAACGTGTC	61.4
12.	Satt449 forward	Left end	GCGTGCTTCTTATATTAGGTGTTAGT	60.1
	Satt449 reverse	Right end	GCGCATTGGAGTTTTTGCTTTT	56.5
13.	Satt565 forward	Left end	GCGCCCGGAACTTGTAATAACCTAAT	63.2
	Satt565 reverse	Right end	GCGCTCTCTTATGATGTTCATAATAA	58.5
14.	Satt277 forward	Left end	GGTGGTGGCGGGTTACTATTACT	62.4
	Satt277 reverse	Right end	CCACGCTTCAGTTGATTCTTACA	58.9
15.	Satt281 forward	Left end	GCGGCGTTAATTTATGCCGGAAA	60.6
	Satt281 reverse	Right end	GCGTTTGGTCTAGAATAGTTCTCA	59.3
16.	Satt267 forward	Left end	CCGGTCTGACCTATTCTCAT	57.3
	Satt267 reverse	Right end	CACGGCGTATTTTTATTTTG	51.2

Table 3: Reaction mixture for PCR amplification

S. No.	Component (conc.)	Final conc.	Single tube (µl)
1.	DNA template	50 ng/µl	2.0 µl
2.	dNTPs (10mM mix)	2.5mM each	1.0 µl
3.	Taq polymerase (5 unit/ µl)	1 unit	0.2 µl
4.	Primer (F+R)	30 ng/µl	1.4 µl
5.	Taq Buffer (10X) + 25 mM MgCl2	1X	1.5 µl
6.	ddW		8.9 µl
		Total	**15 µl**

Scoring of SSR Gel and data analysis

The amplified products were visualized over a transilluminator and photographed under UV light. The amplified products were scored twice manually and independently for each primer. Only clear polymorphic SSR bands of various molecular weight sizes were scored manually in binary formula of 1 or 0 for their presence or absence, respectively, in each lane. All the gels were scored. The binary data was analyzed to obtain Jaccard's similarity coefficients (Jaccard, 1908) among the isolates by using NTSYS-PC (version 2.11W). The SIMQUAL program was used to calculate the Jaccard's similarity coefficients. A common estimator of genetic identity was calculated as follows:

$$\textbf{Jaccard's coefficient} = \frac{N_{AB}}{(N_{AB} + N_A + N_B)}$$

Where, NAB is the number of bands shared by samples, NA represents amplified fragments in sample A, and NB represents fragments in sample B. Similarity matrices based on these indices were calculated. Similarity matrices were used to construct the UPGMA (un-weighted pair group method with arithmetic average) dendrograms to elucidate the diversity among the genotype studied.

RESULT & DISCUSSION

DNA Polymorphism analysis of soybean varieties using SSR markers

Simple sequence repeat markers (SSR) are being extensively used in genome studies, marker assisted selection and cultivar identification and are well known for their versatility in providing a quick assay and for their highly informative data. Twenty SSR markers were screened, of which sixteen (Satt187, Satt228, Satt577, Satt461, Satt146, Satt571, Satt114, Satt268, Satt586, Satt345, Satt300, Satt449, Satt565, Satt277, Satt281, and Satt267) were found suitable and used for assessing molecular diversity among twelve soybean varieties. All the sixteen polymorphic markers were used to generate SSR profiles of 12 varieties. The number of alleles generated by 16 markers for all the twelve varieties were 66, which gave an average of 4.125 alleles per marker. Amplified alleles varied in size from 100 to 500 bp. The high percentage of polymorphic SSR loci detected in this study was similar to the previous studies (Maughan *et al.*, 1995; Rongwen *et al.*, 1995; Diwan and Cregan 1997; Narveletal. 2000; Kumar *et al.*, 2009; Singh *et al.*, 2010; Bisen *et al.*, 2015; Koutu *et al.*, 2019). The summary on markers used and allele amplified are presented in **Table 4.** Gel electrophoresis photographs of SSR marker profile of varieties are given in **figure 1 & 2.**

Table 4: Summary of SSR amplified products in twelve varieties of soybean

Specification	Particular
Total number of markers tested	16
Number of polymorphic markers	16
Total number of monomorphic markers	0
Total number of rare alleles identified	0
Total number of alleles amplified	66
Size range of amplified alleles (in bp)	100 to 500
Average number of alleles per primer	4.125
Total number of polymorphic bands identified	66
Total number of monomorphic bands identified	0
Percentage of all bands that were polymorphic	100

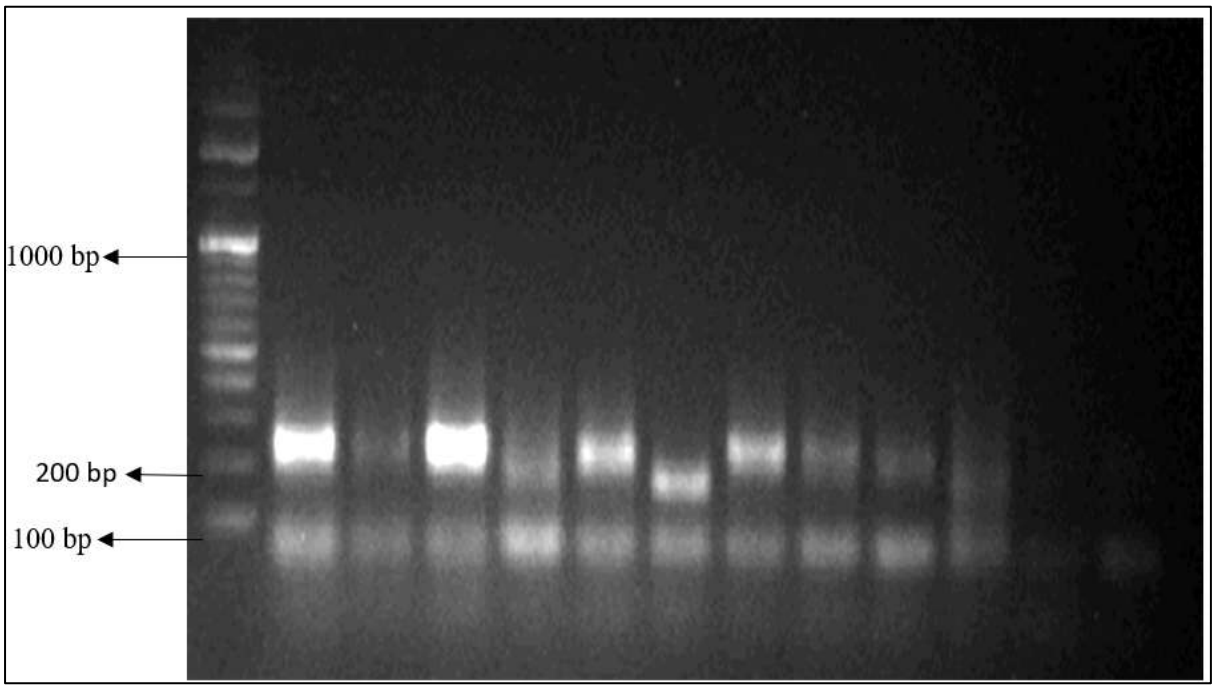

Fig. 1 SSR profile of soybean varieties with primer Satt 281

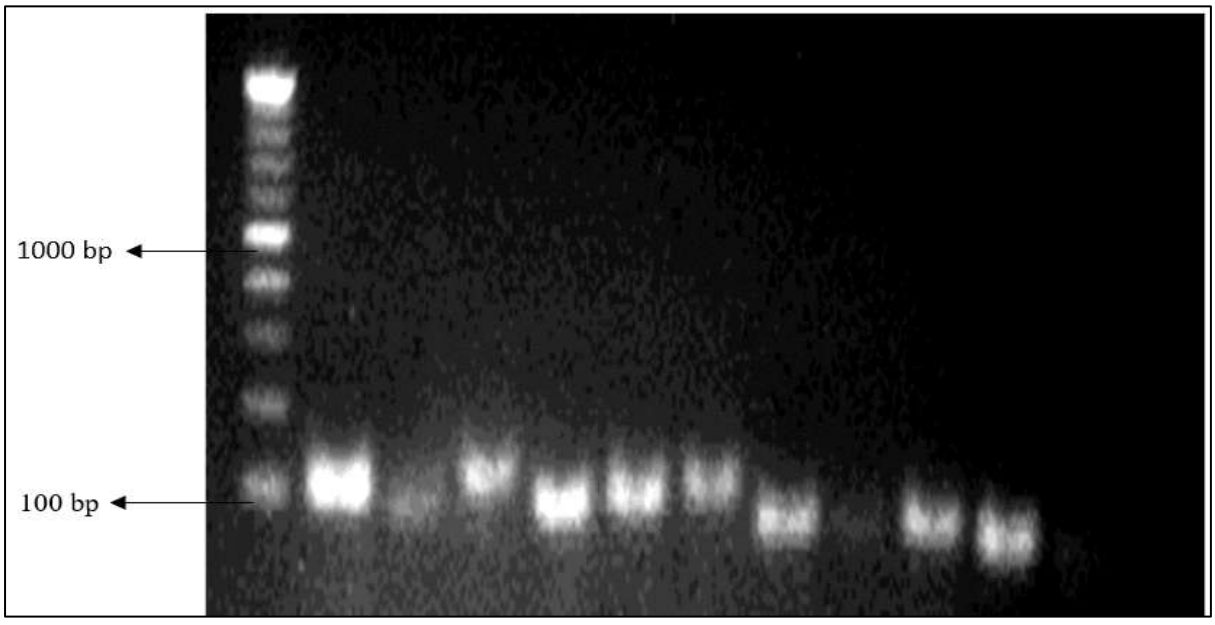

Fig. 2 SSR profile of soybean varieties with primer Satt 114

Genetic relationship among different varieties

The Jaccard's similarity coefficient estimates between pair of different varieties varied from 0.59 [between Kalitur and JS 335, Ankur and Bhatt] to 0.92 [between PK 472 and Ankur] **(Table: 5).** Among the twelve varieties, the six pairs with lowest genetic similarity (GS) value (i.e., maximum diverse pairs) were Kalitur and JS 335; Ankur and Bhatt (0.590 genetic similarity value); Bhatt and PS 1347; PS 1029 and JS 335; Kalitur and PS 1347; and Ankur and PS 1225 (0.621 genetic similarity value). Similarly, pairs with maximum GS value *i.e.,* minimum diversity in the experimental material of the present study was PK 472 and Ankur (0.924 genetic similarity value). For the improvement of crop plants, evaluation of genetic divergence and relatedness among breeding materials has great significance. Knowledge on genetic diversity in soybean could help breeders and geneticists to understand the structure of germplasm, predict which combinations would produce the best offspring and as a result can facilitate the widening of the genetic basis of breeding material for selection. Further information on genetic distances based on microsatellite markers shall be preferred in creating selectable genetic variation using genotypes which are genetically apart (**Panobianco** *et al.*, 2007; Koutu *et al.*, 2019). The diversity analysis can also be utilized for the development of diverse gene pool. The hybridization among the diverse gene pool will result into more heterotic combinations.

Genotype clustering based on molecular data

The UPGMA (un-weighted pair group method with arithmetic mean) dendrogram was constructed using Jaccard's similarity coefficient of SSR marker data of sixteen polymorphic primers generated on twelve varieties employing the program NTSYS 2.11 **(Fig. 3).** The UPGMA ordered the population of twelve varieties into two main groups namely; Group A and Group B. Group A consisted of three varieties forming cluster I and cluster II. Group A differentiated from group B at similarity coefficient of 0.65. Group B contained 9 varieties and was further sub-divided into two subgroups B1 and B2 at 0.669 similarity coefficient value.

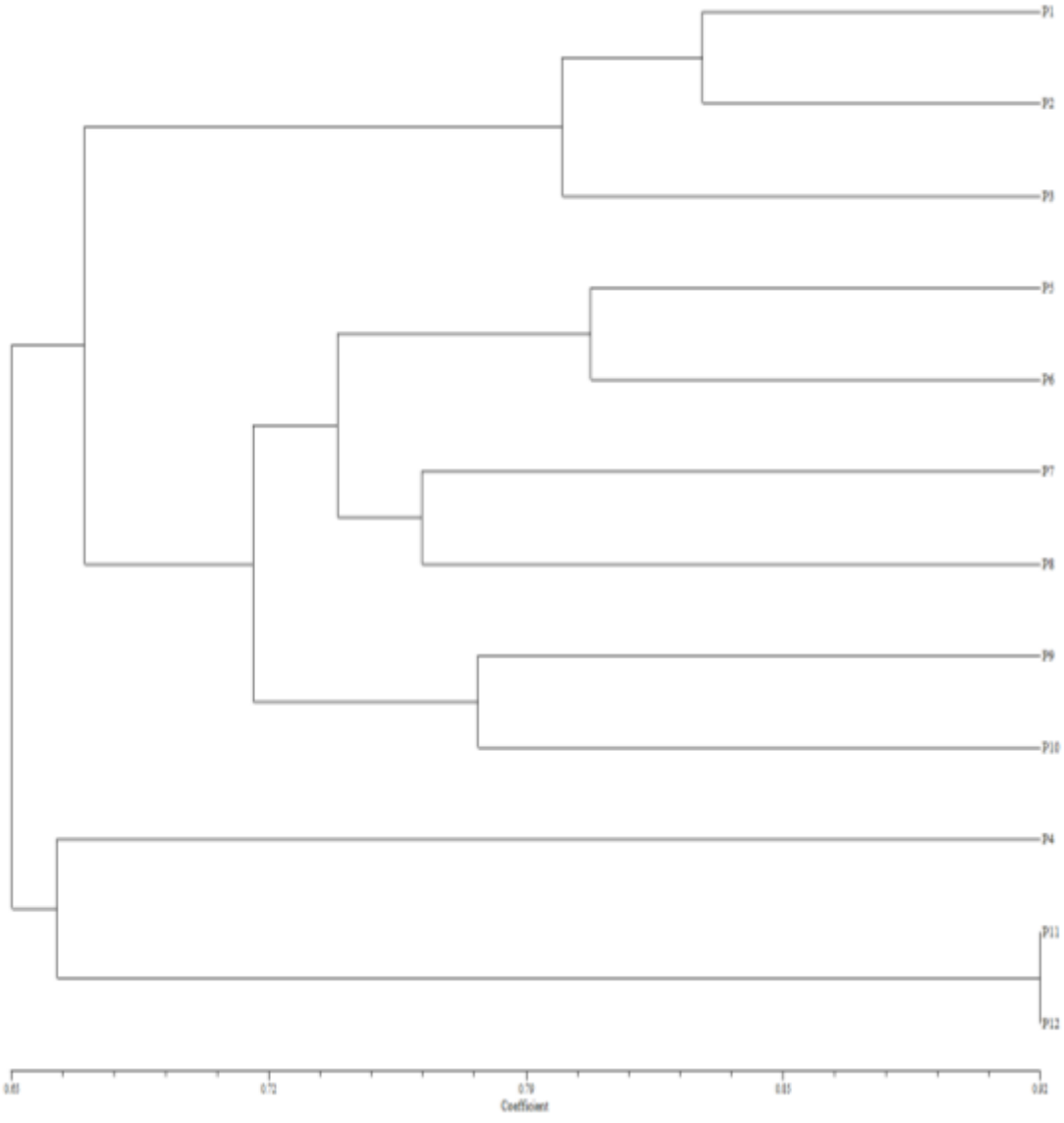

Table 5: Similarity coefficient between varieties using sixteen SSR profiles

1.	1.000											
2.	0.833	1.000										
3.	0.757	0.833	1.000									
4.	0.651	0.636	0.712	1.000								
5.	0.621	0.697	0.682	0.727	1.000							
6.	0.697	0.712	0.667	0.651	0.803	1.000						
7.	0.651	0.667	0.621	0.576	0.727	0.742	1.000					
8.	0.803	0.727	0.682	0.667	0.697	0.773	0.758	1.000				
9.	0.621	0.667	0.591	0.545	0.636	0.742	0.727	0.758	1.000			
10.	0.636	0.652	0.606	0.561	0.652	0.758	0.712	0.712	0.773	1.000		
11.	0.591	0.636	0.652	0.636	0.606	0.652	0.667	0.636	0.667	0.742	1.000	
12.	0.636	0.621	0.636	0.682	0.591	0.667	0.652	0.682	0.652	0.758	0.924	1.000
	1	2	3	4	5	6	7	8	9	10	11	12

B1 and B2 were divided into three (Cluster III and IV) and one cluster (cluster V), respectively. Group A was divided into two clusters at 0.662 similarity coefficient. Cluster I consisted of two varieties; PK 472 and Ankur at 0.92 similarity coefficient. Cluster II consisted of only one variety i.e., Bragg at 0.662 similarity coefficient. Group B was divided into subgroups, B1 and B2. Subgroup B1was further divided into two clusters (III and IV) at 0.71 similarity coefficient. In subgroup B2, one cluster was formed (cluster V). Cluster III consisted of two varieties (PS 1092 and Kalitur) formed at 0.77 Jaccard's coefficient of similarity. Cluster IV had maximum of four varieties namely; Bhatt, PK 327, PS 1029 and PS 1024 with similarity coefficient of 0.73. Cluster V at similarity coefficient value of 0.80 consisted of three varieties; PS 1347, PS 1225 and JS 335. PS 1347 and Ankur were found to be most distant based on dendrogram.

Genetic diversity is critical to crop breeding in cultivar development, germplasm enhancement and end-product commercialization. The genetic structure and genetic diversity of regular grain-type soybean have been well analyzed **(Brown-Guedira *et al.,* 2000; Cui *et al.,* 2001; Ude *et al.,* 2003; Li *et al.,* 2008 and Kaushar, N., 2014)**. Compared to results reported by **Fu *et al.,* (2007),** a higher level of genetic diversity among twelve soybean varieties expressed in terms of genetic similarities was observed in this study. Cluster analysis separated all varieties from each other based on mean genetic similarity coefficient (an arbitrarily line for clustering of genotypes) and assigned them into groups, which largely corresponded to their origin and pedigree. Grouping of varieties revealed congruency of some clusters with the known pedigrees. For example, varieties PS 1024 and PK1029 were clustered together. Similar results were also reported by **Ristova *et al.,* (2010) and Kaushar, (2014)**. **Dayaman *et al.,* (2009)** also clustered 45 soybean accessions according to their place of origin. Low average number of alleles per locus and allelic diversity observed in the set of genotypes analyzed indicated that the genetic base of the material used in the present study is not narrow. Hence, the introduction of new germplasm seems to be essential to ensure sustained progress in future breeding programs.

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