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Evaluation of SRAP marker efficiency to identifying genetic diversity among genotypes of greengram (*Vigna radiata*) (L.)

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Abstract
The genetic variability and relationships among 12 greengram were analyzed using 121 sequencerelated amplified polymorphism (SRAP) markers. 67 out of 121 SRAP primers were found to be the most effective in fingerprinting the greengram genotypes under the study. Out of 362 amplified bands, a total of 307 polymorphic bands were detected which demonstrated with polymorphism information content (PIC) value of 0.695 with a range from 0.142 to 0.906. The distinctive value of SRAP primer index (SPI) for studied markers was 4.149. The unweighted-pair group method arithmetic average (UPGMA) based grouping showed three distinct clusters at a cut-off value of 0.56. In the present study, the average Jaccard similarity coefficient was 0.561 which indicated a moderate level of genetic diversity in studied greengram genotypes. The result of current investigation suggested that there is need to increase the genetic base of greengram germplasm using different breeding approach *viz*, mutagenesis, germplasm introduction, wide hybridization or somaclonal variation.

Keywords: SRAP marker, genetic diversity, greengram

Introduction

Mungbean or greengram (*Vigna radiata*) (L.) Wilczek has been grown in India for a considerable period of time. This popular pulse crop belongs to the family *Leguminaceae*, subgenus Ceratotropis, with a diploid chromosome number of 2n=22 (Smarrt *et al*., 1990) [26]. Mungbean is primarily cultivated in China and India, along with many countries in Southeast Asia. It occupies the third place amongst the pulse crops after pigeonpea and chickpea in this country, covering about 40.38 lakh hectares with the production of 31.5 lakh tons and a productivity rate of 783 kg/ha (Anonymous) [7]. Greengram is one of the crops in which India occupies the leading position in global production, accounting for more than 75%. Plant breeder's rights are of important concern for safeguarding the intellectual property of the plant breeder to continue the development of new varieties. Plant breeding techniques or natural selection has produced varieties that are eligible for certification. However, without a clear strategy for preserving genetic purity, there is a significant risk of losing varietal identity in both scenarios. This highlights the increased need for strategies to maintain plant genetic diversity, particularly through the protection of plant varieties. Morphological descriptors are often used to determine varietal identity, but they can be unreliable because character expression is influenced by environmental factors. Additionally, the limited number of descriptors available makes it difficult to distinguish between the many varieties, underscoring the need for supplementary methods to assist in varietal identification (Silva *et al*., 2012) [24].

The development of sequence-related amplified polymorphism (SRAP) molecular markers by Li and Quiros (2001) [16] has shown promise in the assessment of genetic diversity and in the discrimination of accession. They consist of 17 or 18 nucleotides long core sequences (13–14 bases long), with nonspecific "filler" sequences (the first 10–11 bases beginning at the 5' end) and the sequences CCGG in the forward primer and AATT in the reverse primer following. Two steps of amplification were employed, with five cycles at 35°C and another 35 cycles at the annealing temperature of 50°C. When there are partial matches in the target DNA, efficient primer binding is taken care of by the initial low annealing temperature. Since it was initially reported in 2001, it has become a novel and practical PCR marker technique for various applications such as gene cloning in agricultural plants, molecular mapping, cultivar identification, germplasm characterization, and identification (Aneja *et al*., 2010) [6] (Aneja *et al*., 2012) [4]. For example, SRAP markers can be used for linkage map construction (Yeboah *et al*., 2007) [28], genomic and cDNA fingerprinting, gene tagging Li and Quiros (2001) [16], genetic diversity analysis (Li *et al*., 2009) [17], map-based cloning (Zhang *et al*., 2010) [29], hybrid identification (Kumar Mishra *et al*., 2011) [15], and sex determination (Zhou *et al*., 2011) [30]. Moreover, SRAP markers are more effective than SSR, ISSR, or RAPD markers for revealing genetic diversity among closely related cultivars (Budak *et al*., 2004) [9]. Nonetheless, maintaining varietal integrity and safeguarding against adulteration continue to be crucial responsibilities for the seed industry. The present study allows varietal identification of 12 greengram varieties using SRAP molecular markers.

Materials and Methods

Plant materials

Twelve greengram accessions and their origins are presented in Table 1. Twelve greengram accessions were kindly obtained from Pulse research station, JAU, Junagadh were used in this study.

Table 1: List and source of Greengram accessions used for the current study

Sr. No.	Accessions		Source
1	GJM-2301	GJM-1714 x IPM 409-4	Pulse research station, JAU, Junagadh
2	GJM-2302	SKNM-1606 x IPM 205-7	Pulse research station, JAU, Junagadh
3	GJM-2303	SKNM-1606 x IPM 205-7	Pulse research station, JAU, Junagadh

4	GJM-2304	SKNM-1606 x IPM 205-7	Pulse research station, JAU, Junagadh
5	GJM-2305	IPM 205-7 x GJM-1703	Pulse research station, JAU, Junagadh
6	GJM-2306	IPM 205-7 x GJM-1703	Pulse research station, JAU, Junagadh
7	GJM-2307	IPM 205-7 x GJM-1703	Pulse research station, JAU, Junagadh
8	GM-4	GM-3 x Pusa 9333	Pulse research station, JAU, Junagadh
9	GM-5	-	Pulse research station, JAU, Junagadh
10	GM-6	-	Pulse research station, JAU, Junagadh
11	GM-7	-	Pulse research station, JAU, Junagadh
12	Meha	-	Pulse research station, JAU, Junagadh

DNA Extraction

DNA extraction was carried out using CTAB method. The leaf sample was grinded to a fine powder of extraction buffer was added and mixed and then incubated at 65^0C for 30 min. An equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added for protein precipitation. The samples were centrifuged at 12000 rpm for 15 min at 4^0C. DNA was precipitated using ice-cold isopropanol, and the pellet was washed using 80% ethanol. The quality and concentration of extracted DNA were detected using 0.8% agarose gel electrophoresis. Dilution with TE was carried out.

Sequence-related amplified polymorphism (SRAP) markers

Sequence-related amplified polymorphism (SRAP) markers SRAP-PCR amplification was performed following the methods of Ammar (2015) [2]. The PCR reaction was performed with different combinations of the forward and reverse primers, 50 ng template DNA, and nuclease-free water up to 20 ul. Amplification was carried out on a Variti thermal cycler (Applied Biosystems). The PCR was set with initial denaturation at 94^0C for 5 min followed by five cycles of denaturation at 94^0C for 1 min, annealing at 35^0C for 1 min and elongation at 72^0C for 1 min. In the remaining 30 cycles, the annealing temperature was increased to 50^0C for 1 min with a final extension step at 72^0C for 15 min.

Table 2: SRAP Primer sequence utilized for polymorphism

Primer code	Primer sequence (5'—3') Forward primer	Primer code	Primer sequence (5'—3') Reverse primer
Me01	TGAGTCCAAACCGGATA	Em01	GACTGCGTACGAATTAAT
Me02	TGAGTCCAAACCGGAGC	Em02	GACTGCGTACGAATTTGC
Me03	TGAGTCCAAACCGGAAT	Em03	GACTGCGTACGAATTGAC
Me04	TGAGTCCAAACCGGACC	Em04	GACTGCGTACGAATTTGA
Me05	TGAGTCCAAACCGGAAG	Em05	GACTGCGTACGAATTAAC
Me06	TGAGTCCAAACCGGTAA	Em06	GACTGCGTACGAATTGCA
Me07	TGAGTCCAAACCGGTCC	Em07	GACTGCGTACGAATTCAA
Me08	TGAGTCCAAACCGGTGC	Em08	GACTGCGTACGAATTCTG
Me09	TGAGTCCAAACCGGACG	Em09	GACTGCGTACGAATTCGA
Me10	TGAGTCCAAACCGGACT	Em10	GACTGCGTACGAATTCAG
Me11	TGAGTCCAAACCGGAGG	Em11	GACTGCGTACGAATTCCA

Separation and photographing of PCR products

PCR products were separated on 1.8% agarose gel in 1X TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA) at 115 volt for 1.5-2 h. A 100 bp standard DNA ladder as the molecular standard marker was used to confirm the appropriate (SRAP bands). The banding patterns were photographed under UV light for further analysis.

Molecular data analysis

Scoring gels and data analyses each band was scored as present (1) or absent (0) and data were analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) software package (Rohlf, 1998) [21]. A similarity matrix was constructed based on Jaccard's coefficient (Jaccard, 1908) [13], which considers only one to one matches between two taxa for similarity. The similarity matrix was used to construct a dendrogram using the UPGMA (unweighted-pair group method arithmetic average) to determine genetic relationships among the germplasm studied. Unlike molecular markers that show binary distribution, phenotypic marker data have continuous distribution for most traits. Therefore, row data were first standardized using the standardization option nested in NTSYS-pc software package. In order to see how well a cluster analysis represents the distance matrix, COPH module was used to transform the tree matrix to a matrix of ultrametric distances (a matrix of distances implied by the cluster analysis). Finally, MXCOMP module was used to compare these ultrametric distances and distance matrix produced for UPGMA analysis. The polymorphism information content (PIC) for each primer was calculated to estimate its allelic variation as follows: $PIC_i=1-\sum P_{ij}^2$, where P_{ij} is the frequency of the ith allele for marker j and the summation extends over n alleles, calculated for each SRAP marker (Anderson *et al*., 1993) [3].

Result and Discussion

Banding profile of the genotypes under study is listed in Table 3. Keeping genotype specific amplification pattern (Fig 1), a diagrammatic mode of DNA fingerprint comprising of genotype specific bands generated by specific primers was developed. The presence of band was scored as 1 and absence as 0. A critical perusal indicated that 67 out of 121 SRAP primers were most useful in fingerprinting greengram genotypes under study. A set of 67 SRAP primer combinations revealed a high polymorphism percentage of 100% (representing the profiles of 307 polymorphic bands from 362 scorable bands (Table 3). However, Aneja (2013) [5] and Alghamdi (2017) [1] found 100% polymorphism among Indian mungbean varieties and Saudi mungbean germplasms, respectively. Although we obtained a comparatively lower polymorphism percentage than those of previous studies, our study used higher numbers of primer combinations and scorable DNA bands, both of which may affect polymorphism percentage. Each primer combination generated an average of 4.5 bands, of which 20 exhibited polymorphism. The lowest number of polymorphic bands was achieved with the primer combination SRAP-03 and SRAP-04 (1) with polymorphism percentage of 50. Whereas, the primer combinations SRAP-58 generated the highest numbers of polymorphic bands (Zhou *et al*., 2011) [30]. Highest polymorphism percentage (100%) found in 37 out of 67 primer combinations. From all primer combinations, an average polymorphism percentage of 83.4% was revealed across all genotypes. This was comparable to that found in mungbean and blackgram (89.51%) using inter-simple sequence repeat (ISSR) markers (Tantasawat *et al*., 2010) [27]. However, the polymorphism levels may be restricted by the self-pollinated nature of these two species. The number of amplicons amplified ranged from 2 to 11 with a mean of 5.4 amplicons per primer combination which is higher than SRAP markers-based study in okra (Gulsen *et al*., 2017) [11]. This suggests the presence of substantial polymorphism at studied SRAP loci. Due to variation in primer binding sites each primer gave different number of amplicons. The coefficient cophenetic value with Mantel's test reached 0.902 ($p < 0.01$), indicating high reliability of distance matrix data represented in the dendrogram (fig. 2) (Mantel *et al*., 1967) [18].

Among the all pair-wise combinations of genotypes, Jaccard's similarity coefficients based on SRAP markers ranged from 0.326 to 0.796 with an average value of 0.561. The similarity coefficients values were highest between the accessions GM-6 and GM-7 (0.796) and lowest between the accessions GJM-2302 and GM-7 (0.326) Table 4.

The PIC values for each primer are most often analyzed to estimate discriminatory power by accounting both the number of alleles at a locus and their relative frequencies (Nagl *et al*., 2011) [20]. The highest (0.906) PIC value through SRAP fingerprinting data was recorded for primer SRAP-58 and the lowest (0.142) for SRAP-4. The average PIC value was found to be 0.695 (Table 3). This marker efficiently discriminated the different genotypes of greengram. DNA fingerprinting is a routine method employed to study the extent of genetic diversity across a set of genotypes or cultivars and group them into specific categories. Comparative studies in Vigna species involving RAPD, AFLP, ISSR, and SSR marker systems were successfully used and reported by researchers (Gunjeet Kaur *et al*., 2016) [12] however, the reports on greengram are very limited. The discriminative power of DNA markers used as tools to characterize greengram is very important because they can be used to assess the genetic diversity among the genotypes of greengram. In the present study, we used three different molecular marker systems along with morphological markers to define genetic relationships between greengram genotypes with high levels of polymorphism, consistent with earlier reports with different marker systems (Muthusamy *et al*., 2008) [19] and (Singh *et al*., 2013) [25]. The highest (9.966) SRAP primer index (SPI) value through SRAP fingerprinting data was recorded for primer SRAP-58 and the lowest (0.284) for SRAP-4. The average SPI value was found to be 4.149 (Table 3). In present study obtained highest PIC and SPI values than the previous study. The average marker index for RAPD was 2.44, followed by ISSR with 1.09 and SSR with 0.318 (Gunjeet Kaur *et al*., 2016) [12]. Therefore, the use of this marker system can reduce cost, time, and labor in the identification of varieties of mungbean and blackgram. Tantasawat, (2010) [27] obtained similar findings when using only six most informative ISSR primers. However, genetic identification based on SRAP loci is more likely to reflect phenotypic trait expressions than other multilocus markers including ISSR markers, which are largely located in non-coding genomic regions (Shao *et al*., 2010) [23].

In present investigation, 67 SRAP primer combinations amplified 362 bands, of which 307 were polymorphic (Table 3). The extent polymorphism is important to identify accurate associations among accessions. The level of polymorphism was comparatively higher than previous studies on many crops *viz*., 82.5% in buckwheat (Li *et al*., 2009) [17] and 55% in Pinus (Feng *et al*., 2009) [10]. The polymorphism level of SRAP in cumin was also higher than the report of Rostami-Ahmadvandi (2013) [22] where ISSRs and 13 RAPDs revealed 67.32% and 54.90% polymorphism, respectively. This difference may be due to amplification of both coding and non-coding regions of genome by RAPD and ISSR markers while SRAP markers amplify only coding region (Bargish TA and Rahmani F, 2016) [8]. The polymorphism value of this study was also higher than AFLP based study in cumin (Kermani *et al*., 2006) [14]. High marker polymorphism indicated the prevalence of good diversity among studied accessions.

A phylogenetic tree revealed the genetic relatedness existing among the greengram genotypes based on the SRAP marker as shown by the bootstrap values. The 12 genotypes were grouped into two main clusters (A and B), at similarity index of 56%. Which were further subdivided into four sub-clusters. The Clusters I and II comprised 4 and 6 genotypes, respectively. Main cluster A comprised ten genotypes that were further subdivided to form two sub clusters. Sub cluster I comprised namely, GJM-2301, GJM2303, GJM-2304 and GJM-2306 and GJM-2307, GM-6, GM-7 GAM-5, MEHA and GM-4 genotypes included in sub cluster II. GJM-2302 genotype falls alone in sub cluster III. Genotype GJM-2305 formed its own sub cluster IV. Sub cluster III and IV both related to main cluster A at similarity index of 58%.

Table 3: Size, number of amplified bands, per cent polymorphism, PIC and SPI obtained by SRAP primers in 12 greengram varieties

Sr. no.	Combinations	Primers	Total no of bands	Monomorphic Bands	Polymorphic Bands	Polymorphism %	PIC	SPI
1	Me-01 Em-01	SRAP-01	2	0	2	100.0	0.245	0.490
2	Me-01 Em-02	SRAP-02	7	4	3	42.9	0.841	5.887
3	Me-01 Em-03	SRAP-03	2	1	1	50.0	0.320	0.640
4	Me-01 Em-05	SRAP-04	2	1	1	50.0	0.142	0.284
5	Me-01 Em-06	SRAP-05	3	1	2	66.7	0.653	1.959
6	Me-01 Em-07	SRAP-06	10	1	9	90.0	0.890	8.900
7	Me-01 Em-08	SRAP-07	7	4	3	42.9	0.844	5.908

8	Me-01 Em-09	SRAP-08	9	0	9	100.0	0.855	7.695
9	Me-01 Em-10	SRAP-09	6	0	6	100.0	0.771	4.626
10	Me-01 Em-11	SRAP-10	4	2	2	50.0	0.711	2.844
11	Me-02 Em-01	SRAP-11	7	1	6	85.7	0.815	5.705
12	Me-02 Em-02	SRAP-12	4	0	4	100.0	0.732	2.928
13	Me-02 Em-04	SRAP-13	5	0	5	100.0	0.699	3.495
14	Me-02 Em-05	SRAP-14	3	1	2	66.7	0.615	1.845
15	Me-02 Em-06	SRAP-15	10	0	10	100.0	0.870	8.700
16	Me-02 Em-07	SRAP-16	10	2	8	80.0	0.876	8.760
17	Me-02 Em-09	SRAP-17	2	0	2	100.0	0.198	0.396
18	Me-02 Em-10	SRAP-18	7	2	5	71.4	0.846	5.922
19	Me-02 Em-11	SRAP-19	6	0	6	100.0	0.870	5.220
20	Me-03 Em-01	SRAP-20	4	0	4	100.0	0.750	3.000
21	Me-03 Em-02	SRAP-21	3	1	2	66.7	0.272	0.816
22	Me-03 Em-04	SRAP-22	3	1	2	66.7	0.255	0.765
23	Me-03 Em-05	SRAP-23	5	1	4	80.0	0.725	3.625
24	Me-03 Em-11	SRAP-24	2	0	2	100.0	0.498	0.996
25	Me-04 Em-01	SRAP-25	6	1	5	83.3	0.806	4.836
26	Me-04 Em-09	SRAP-26	5	3	2	40.0	0.798	3.990
27	Me-04 Em-10	SRAP-27	6	0	6	100.0	0.771	4.626
28	Me-04 Em-11	SRAP-28	2	2	0	0.0	0.500	1.000
29	Me-05 Em-01	SRAP-29	3	0	3	100.0	0.615	1.845
30	Me-05 Em-02	SRAP-30	4	0	4	100.0	0.669	2.676
31	Me-05 Em-04	SRAP-31	4	1	3	75.0	0.699	2.796
32	Me-06 Em-02	SRAP-32	9	0	9	100.0	0.853	7.677
33	Me-06 Em-04	SRAP-33	5	0	5	100.0	0.770	3.850
34	Me-06 Em-06	SRAP-34	6	2	4	66.7	0.792	4.752
35	Me-06 Em-07	SRAP-35	8	0	8	100.0	0.839	6.712
36	Me-06 Em-08	SRAP-36	4	1	3	75.0	0.566	2.264
37	Me-06 Em-09	SRAP-37	5	0	5	100.0	0.751	3.755
38	Me-06 Em-10	SRAP-38	2	0	2	100.0	0.480	0.960
39	Me-06 Em-11	SRAP-39	3	0	3	100.0	0.653	1.959
40	Me-07 Em-01	SRAP-40	5	0	5	100.0	0.714	3.570
41	Me-07 Em-02	SRAP-41	6	1	5	83.3	0.830	4.980
42	Me-07 Em-03	SRAP-42	5	0	5	100.0	0.760	3.800
43	Me-07 Em-04	SRAP-43	4	4	0	0.0	0.750	3.000
44	Me-07 Em-05	SRAP-44	4	0	4	100.0	0.639	2.556
45	Me-07 Em-06	SRAP-45	6	6	0	0.0	0.833	4.998
46	Me-07 Em-07	SRAP-46	11	2	9	81.8	0.897	9.867
47	Me-07 Em-08	SRAP-47	6	0	6	100.0	0.826	4.956
48	Me-07 Em-10	SRAP-48	6	1	5	83.3	0.818	4.908
49	Me-07 Em-11	SRAP-49	5	0	5	100.0	0.738	3.690
50	Me-08 Em-01	SRAP-50	2	0	2	100.0	0.488	0.976
51	Me-08 Em-04	SRAP-51	8	2	6	75.0	0.836	6.688
52	Me-08 Em-05	SRAP-52	6	2	4	66.7	0.750	4.500
53	Me-08 Em-06	SRAP-53	2	0	2	100.0	0.500	1.000
54	Me-08 Em-07	SRAP-54	4	0	4	100.0	0.745	2.980
55	Me-08 Em-08	SRAP-55	8	0	8	100.0	0.838	6.704
56	Me-08 Em-11	SRAP-56	5	1	4	80.0	0.646	3.230
57	Me-09 Em-01	SRAP-57	9	1	8	88.9	0.864	7.776
58	Me-09 Em-02	SRAP-58	11	0	11	100.0	0.906	9.966
59	Me-09 Em-03	SRAP-59	8	0	8	100.0	0.831	6.648
60	Me-09 Em-04	SRAP-60	7	0	7	100.0	0.832	5.824
61	Me-09 Em-05	SRAP-61	5	0	5	100.0	0.769	3.845
62	Me-09 Em-07	SRAP-62	4	0	4	100.0	0.710	2.840
63	Me-09 Em-08	SRAP-63	4	0	4	100.0	0.667	2.668
64	Me-11 Em-04	SRAP-64	5	1	4	80.0	0.723	3.615
65	Me-11 Em-09	SRAP-65	7	0	7	100.0	0.832	5.824
66	Me-11 Em-10	SRAP-66	6	1	5	83.3	0.782	4.692
67	Me-11 Em-11	SRAP-67	8	0	8	100.0	0.854	6.832
	Total		362	55	307			
	Average		5.402985075		4.582089552	83.16264995	0.704973	4.149806
							0.906	9.966
							0.142	0.284

Table 4: Dice similarity coefficient of 12 Greengram accessions based on SRAP data analysis

Row\Col	GJM-2301	GJM-2302	GJM-2303	GJM-2304	GJM-2305	GJM-2306	GJM-2307	GM-4	GAM-5	GM-6	GM-7	MEHA
GJM-2301	1.000											
GJM-2302	0.408	1.000										
GJM-2303	0.619	0.466	1.000									
GJM-2304	0.604	0.522	0.623	1.000								
GJM-2305	0.419	0.575	0.464	0.606	1.000							
GJM-2306	0.569	0.492	0.606	0.756	0.530	1.000						
GJM-2307	0.615	0.372	0.575	0.582	0.376	0.573	1.000					
GM-4	0.565	0.420	0.450	0.628	0.465	0.611	0.676	1.000				
GAM-5	0.598	0.386	0.557	0.569	0.395	0.559	0.724	0.697	1.000			
GM-6	0.619	0.373	0.569	0.559	0.367	0.550	0.773	0.623	0.741	1.000		
GM-7	0.569	0.326	0.529	0.502	0.334	0.519	0.699	0.565	0.701	0.796	1.000	
MEHA	0.558	0.342	0.493	0.479	0.321	0.491	0.667	0.590	0.668	0.709	0.750	1.000

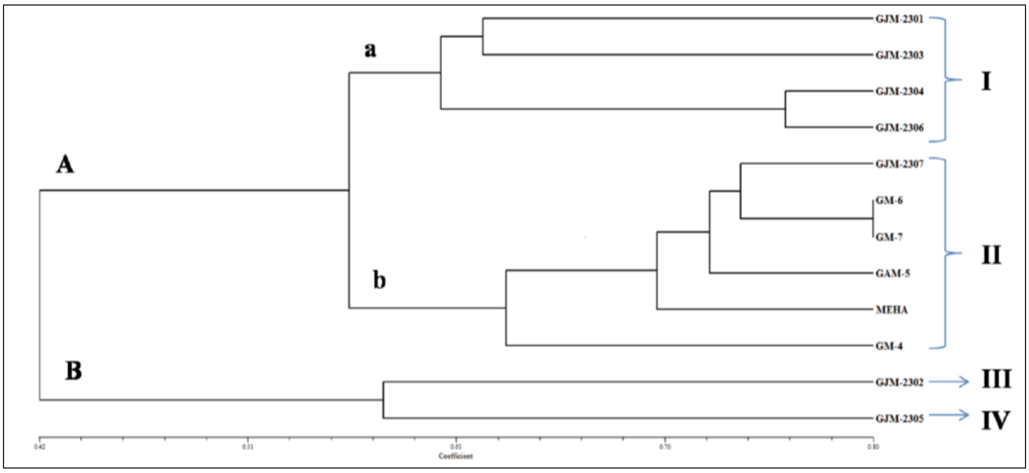

Fig 1: Dendrogram produced by Jaccard's coefficient and the unweighted pair group method with arithmetic average (UPGMA) clustering method based on SRAP data in 12 greengram accessions

Conclusion

The SRAP marker system is a simple, efficient marker system that can be easily use in crops for various purposes, ranging from map construction to gene tagging, genomic and cDNA fingerprinting, and map-based cloning. This system offers several advantages over other systems, which include simplicity, reasonable throughput rate, discloses numerous codominant markers, allows easy isolation of bands for sequencing, and most importantly, targets ORFs. Results show there is a moderate level of genetic variability among elite greengram genotypes. SRAP markers are, therefore, very useful for assessing greengram diversity and selecting a core collection to enhance the efficiency of genotype management for use in greengram breeding and conservation. Characterization and assessment of diversity among the greengram genotypes are very essential in designing breeding strategies for qualitative and quantitative traits. In this investigation, levels of inter- and intra-specific diversity relationships among different genotypes of greengram have been assessed. The results drawn from this present study would be of high use in greengram breeding programs and may be used further for crop improvement using advanced marker systems. Incorporation of the molecular approaches with the conventional techniques is genetically more informative.

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