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Full Length Research Paper

Genetic diversity as assessed by morphological and microsatellite markers in greengram (*Vigna radiata* L.)

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Genetic diversity in 20 elite greengram [*Vigna radiata* (L.) R. Wilczek] genotypes were studied using morphological and microsatellite markers. 18 morphological traits generated 50 alleles and the number of alleles ranged from two to five. 16 microsatellite markers from greengram, adzuki bean, common bean and cowpea were successfully amplified across 20 greengram genotypes of which 14 showed polymorphism. A total of 41 alleles were generated with an average of 2.56 alleles per locus. The number of alleles ranged from two to five. Similarity measures and clustering analyses were made using 91 alleles generated from morphological and microsatellite data. The resulting dendrogram distributed 20 greengram genotypes into six main clusters. The results of the principal component analysis were well congruent with the dendrogram. In the dendrogram as well as in the principal component analyses, genotype *Vigna umbellata* was placed separately from the rest of the genotypes. There was successful cross species amplification of adzuki bean, common bean and cowpea microsatellite or simple sequence repeat (SSR) markers in greengram. This study demonstrates that a combination of morphological and molecular markers increases the efficiency of diversity measured and the adzuki bean microsatellite markers are highly polymorphic and can be successfully used for genome analysis in greengram. Results indicate that sufficient variability is present in the greengram genotypes studied and would be helpful in the selection of suitable parents for breeding purposes and gene mapping studies.

Key words: Greengram, *Vigna radiata*, morphological markers, cross species amplification, microsatellite markers, genetic diversity.

INTRODUCTION

Pulses are referred to as the 'poor man's meat' and 'rich man's vegetable'. Greengram [*Vigna radiata* (L.) R. Wilczek] is one of the important pulse crops grown in India. It belongs to the subgenus *Ceratotropis* in the genus *Vigna* and is a self pollinating diploid grain legume (2n = 22) with a genome size of 560 Mb (Arumuganathan and Earle, 1991). With its high protein content (22 to 28%), greengram is a major source of dietary protein for the predominantly vegetarian population of India. The area under greengram in the India is around 3.8 million hectares with a production of 1.0 million tonnes (Anonymous, 2010). In recent years, there has been significant decline in the pulse production in India. Lack of suitable varieties and genotypes with adaptation to local conditions is among the factors affecting the production. In the absence of distinct morphological attributes, genetic characterization of diverse genotypes and documentation of diversity among the genotypes are of utmost significance in genetic improvement of this important legume species (Sivaprakash et al., 2004).

For selection of genetically diverse parents from the germplasm, it is essential that the collection should be systematically characterized using descriptors encompassing both quantitative and qualitative traits.

Qualitative characters are important for plant description and are influenced by consumer's preference, socio-

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Abbreviations: SSR, Simple sequence repeat; **UPGMA,** unweighted pair group method and arithmetic average.

Table 1. List of greengram genotypes with pedigree used in the study.

S/N	Variety	Parentage	Special feature
1.	Rajendran	Selection from Jayankondam local	Tolerant to drought
2.	ADT 2	ADT 1 x AB 33	Resistant to powdery mildew, suitable for rice fallow
3.	K1	Co 4 × ML 65	Tolerant to drought. Suitable for pure and cotton based intercropping systems
4.	KM 1	S.8 x PS 16	Tolerant to Yellow Mosaic Virus (YMV) and pod borer
5.	ADT 3	(H 70 - 16 x Rajendran) x G.65	Resistant to YMV and stemfly.
6.	Vamban 1	Hybrid derivative of S.8 x PIMS 3	High yield, tolerant to YMV
7.	Co 2	Pureline selection from PLS 365/3	Suitable for irrigated condition
8.	VRM (Gg)1	Selection from K851	Location specific variety. Suited to Vellore and Tiruvannamalai districts.
9.	Co 5	KM 2 x MG 50-10	High biomass, resistant to tip blight, tolerant to powdery mildew and root rot. Suitable for rainfed cropping.
10.	Co 4	Gamma irradiated mutant of Co 1 (20 kR)	High biomass, suitable for rainfed condition
11.	Co 3	Pureline selection from PLS 362/2	Suitable for rainfed condition , tolerant to root rot and yellow mosaic virus
12.	CGG 934	Co 6 x WGG 37	Resistant to YMV
13.	CGG 936	Pusa bold x Co 6	Resistant to YMV
14.	CGG 923	Co 5 x WGG 37	Resistant to YMV
15.	Paiyur 1	Pureline selection form DPT 703	Higher grain yield, low incidence of YMV
16.	Co 6	WGG 37 x Co 5	Resistant to yellow mosaic virus, suited to all greengram growing tracts of Tamil Nadu
17.	Co(Gg) 7	MGG 336 x CoGG 902	High yielder, short duration, high protein content (25.2%). Seeds are medium bold with good cooking quality.
18.	KM 2	No.122 x S.9	Tolerant to YMV and pod borer
19.	VBN (Gg) 2	VGG 4 x MH 309	Shiny grains, lobed leaves and resistant to YMV
20.	*Vigna umbellata*	Wild species	Resistant to biotic and abotic stresses

economic scenario and natural selection. Recently, new molecular genetic techniques have increasingly been used to describe plant germplasm collections. In contrast to morphological traits, molecular markers can reveal differences among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, conservation and management (Tanksley et al., 1989). Limited work has been done so far with nuclear DNA diversity in greengram.

Microsatellites or simple sequence repeat (SSR) markers are short tandem repetitive DNA sequences with a repeat length of a few (one to five) base pairs. Microsatellites have become the molecular markers of choice for a wide range of applications such as genetic mapping and genome analysis, genotype identification and variety protection, seed purity evaluation and germplasm conservation, diversity studies, paternity determination and pedigree analysis, gene and quantitative trait locus analysis and marker-assisted breeding (Archana and Jawali, 2007). A narrow genetic base has been reported for the released Indian greengram cultivars using randomly amplified polymorphic DNA (RAPD) (Lakhanpaul et al., 2000) and amplified fragment length polymorphism (AFLP) (Bhat et al., 2005) markers. Paucity of polymorphic molecular markers in greengram has been a limiting factor in application of molecular tools for its genetic improvement. Transfer of markers from other related species offers an alternative option to increase the number of available markers. The present study was aimed to assess the genetic diversity of elite genotypes of greengram using morphological and microsatellite markers and attempt to amplify other legume species SSRs in greengram.

MATERIALS AND METHODS

Plant materials and deoxyribonucleic acid (DNA) extraction

The experimental material for this study comprised of 19 genotypes of greengram released for commercial cultivation over a period of 40 years and *Vigna umbellata*, a wild species (out group) was maintained in the Department of Pulses, Tamil Nadu Agricultural University, Coimbatore. Greengram genotypes along with the pedigree and useful characters are listed in Table 1. These genotypes contain many agronomically important

Table 2. Particulars of morphological traits used in the study.

S/N	Morphological trait	Number of alleles
1	Stem colour	2
2	Petiole colour	2
3	Growth habit	2
4	Raceme position	2
5	Leaf pubescence	2
6	Seed shape	3
7	Pod pubescence	3
8	Leaf colour	3
9	Calyx colour	2
10	Pod attachment to peduncle	3
11	Corolla colour	4
12	Terminal leaflet shape	4
13	Immature pod colour	2
14	Colour of ventral suture of immature pod	3
15	Pod colour at mature stage	4
16	Pod beak shape	2
17	Seed colour	5
18	Lusture on seed surface	2
Total		50
Mean		2.78

characters like resistance to biotic and abiotic stresses and thus are very useful for the improvement of greengram. DNA was extracted from overnight soaked seeds using modified cetyl trimethylammonium bromide (CTAB) method. The concentration of the DNA samples was determined by a spectrophotometer and DNA samples were diluted to 50 ng/µl for polymerase chain reaction (PCR) amplification.

Morphological traits

All the 20 genotypes were raised at the experimental farm in a randomized block design with three replications. 18 morphological characters listed in the National Bureau of Plant Genetic Resources (NBPGR) minimal descriptors for greengram were recorded in each genotype per replication.

Simple sequence repeat (SSR) amplification

SSR amplification reactions were carried out in a volume of 15 µl consisted of 50 ng of template DNA, 0.5 µM of each primer (forward and reverse), 0.15 units of *Taq* DNA polymerase, 2.5 mM of each dNTP, 1 × reaction buffer and distilled de-ionized water. The reaction began with an initial denaturation step of 3 min at 94°C, followed by 35 cycles at 94°C for 45 s, 47 to 57°C (depending upon the primer pair) for 1 min, 72°C for 1 min and 1 cycle of 72°C for 10 min. PCR amplified products were subjected to electrophoresis in a 3% agarose gel in 1 x Tris-borate EDTA (TBE) buffer at 90 v for 3 h. The ethidium bromide stained gels were documented using Alpha Imager ^{TM} 1200 (Alpha Innotech Corporation, California, USA). SSR primers published by Li et al. (2001) in cowpea, Blair et al. (2003) in common bean, Gwag et al. (2006) in greengram and Chaitieng et al. (2006) in adzuki bean were used in this study.

Data analysis

Both morphological traits and SSR markers were scored visually for their presence (1) or absence (0). To measure the informativeness of the markers, the polymorphism information content (PIC) for each SSR locus was calculated according to the formula (Weir, 1996): PIC = 1- ($\sum pi^2$), where, i is the total number of alleles detected for a SSR marker, and pi is the frequency of the i^{th} allele in the set of the 20 genotypes investigated. A dendrogram was constructed based on Jaccard's similarity coefficient (with unweighted pair group method and arithmetic average (UPGMA) using the NTSYS-pc version 2.02 (Rohlf, 2000). The marker data was then standardized for principal component analysis (PCA) using NTSYS-pc software to highlight the resolving power of ordination.

RESULTS AND DISCUSSION

Morphological characteristics provide the basic information about the magnitude of genetic variability. 18 morphological characters were recorded in 20 greengram genotypes. Using 18 morphological traits, 50 alleles were generated that could be scored with an average of 2.78 alleles per trait (Table 2). The number of alleles ranged from two to five. The seed colour generated five alleles and the other traits corolla colour, terminal leaflet shape and mature pod colour generated four alleles per trait.

24 SSR primer pairs were used for analyzing the polymorphism in greengram, of which 16 primers resulted in amplification. Amplification of genomic DNA of the 20 genotypes using 16 SSR primers produced 41 alleles that could be scored with an average of 2.56 alleles per primer (Table 3). The number of amplified alleles ranged from two to five and which varied in size from 110 to 350 bp. Two primers MB77 and CP37 were monomorphic among the genotypes studied. The PIC value ranged from 0.500 to 0.889 which was in close agreement with earlier reported by Gupta and Gopalakrishna (2009) using adzukibean derived SSR markers in blackgram.

Alleles generated from morphological (50) and SSR analyses (41) were combined for UPGMA cluster analysis. The 20 genotypes were grouped into six clusters with a Jaccard's similarity coefficient ranging from 0.26 to 0.79. The dendrogram comprising of the genotypes grouped into different clusters is shown in the Figure 1. High similarity coefficient (0.79) was observed between pre release cultures CGG 923 and CGG 936. An out group genotype *V. umbellata* was the most distinct among the genotypes. The result of PCA analysis was comparable to the cluster analysis (Figure 2). *V. umbellata* appears very distinct from other genotypes in the PCA. The genotype KM1 was grouped separately and CGG 923 and CGG 936 placed closer in the PCA.

A prerequisite for improving the overall characteristics is an understanding of the structure of the germplasm collection. This in turn will allow a systematic sampling of the germplasm for breeding and conservation purpose. Many of the time, morphological traits remain the basis for genetic diversity analysis. Microsatellite markers have been used to quantify genetic diversity and determine

Table 3. Particulars of SSR primer pairs used in the study.

S/N	SSR primer pair	Primer sequence (5' – 3')	Annealing temperature (°C)	Number of alleles	Allele size range (bp)	PIC value
1	MB 7	F: CTTGCTTGCGAGGATGAC R: TCCAGTGCAGCAGATTGA	53	2	290 - 350	0.500
2	MB 13	F:GCAGCAACAACAGCAACA R:GCAGGTTTTGTGGCTCAG	57	2	190 - 195	0.525
3	MB 14	F:TGGAATTTGGAAGGAAGGA R:GATGCAGGTGTTTGGGAG	57	3	175 - 185	0.889
4	MB 17	F:ACCTGCAAGTTGGCAAGA R:TATGTGCACGCATGGAAG	57	2	164 - 166	0.500
5	MB77	F:GGAGAGGAAGGAACAGGG R:GGCAGAGCATAACATGGC	57	1	300	0.000
6	MB 87	:TCCCTTGTGGGAGATCCT R:CTTTGCCACACTCCTTGC	57	2	290 - 293	0.500
7	MB 91	F:GAGGCCAATCCCATAACTTT R:AGCACCACATCAGAGATTCC	57	3	170 - 175	0.667
8	AB 6	F:AATTGCTCTCGAACCAGCTC R:GGTGTACAAGTGTGTGCAAG	53	4	110 - 130	0.750
9	AB 8	F:AGGCGAGGTTTCGTTTCAAG R:GCCCATATTTTTACGCCCAC	55	4	116 - 120	0.750
10	AB 10	F:TGGGCTACCAACTTTTCCTC R:TGAGCGACATCTTCAACACG	53	2	195 - 198	0.500
11	AB 14	F:GCTTGCATCACCCATGATTC R:AAGTGATACGGTCTGGTTCC	53	5	150 - 200	0.800
12	AB 27	F:ACTGGATGAGGGTTTAGTGCG R:CTGTCTTGTCTTGTGGGTTCGTTC	57	2	110 - 112	0.500
13	AB 50	F:TCCCACTTCTCCATTACCTCCAC R:GAGATTATCTTCTGGGCAGCAAGG	55	2	120 - 124	0.500
14	CB 2	F;GCGACAGCAAGAGAACCTC R:CAACAAACGGTGATTGACCA	47	3	108 - 110	0.667

Table 3. Contd.

15	CP 24	F:TCAACAACACCTAGGAGCCAA R:ATCGTGACCTAGTGCCCACC	57	3	145 - 155	0.667
16	CP37	F:TGTCCGCGTTCTATAAATCAGC R:CGAGGATGAAGTAACAGATGATC	53	1	290	0.000
Total				41	-	8.716
Mean				2.56	-	0.544

MB, Mungbean; AB, adzukibean; CB, commonbean; CP, cowpea.

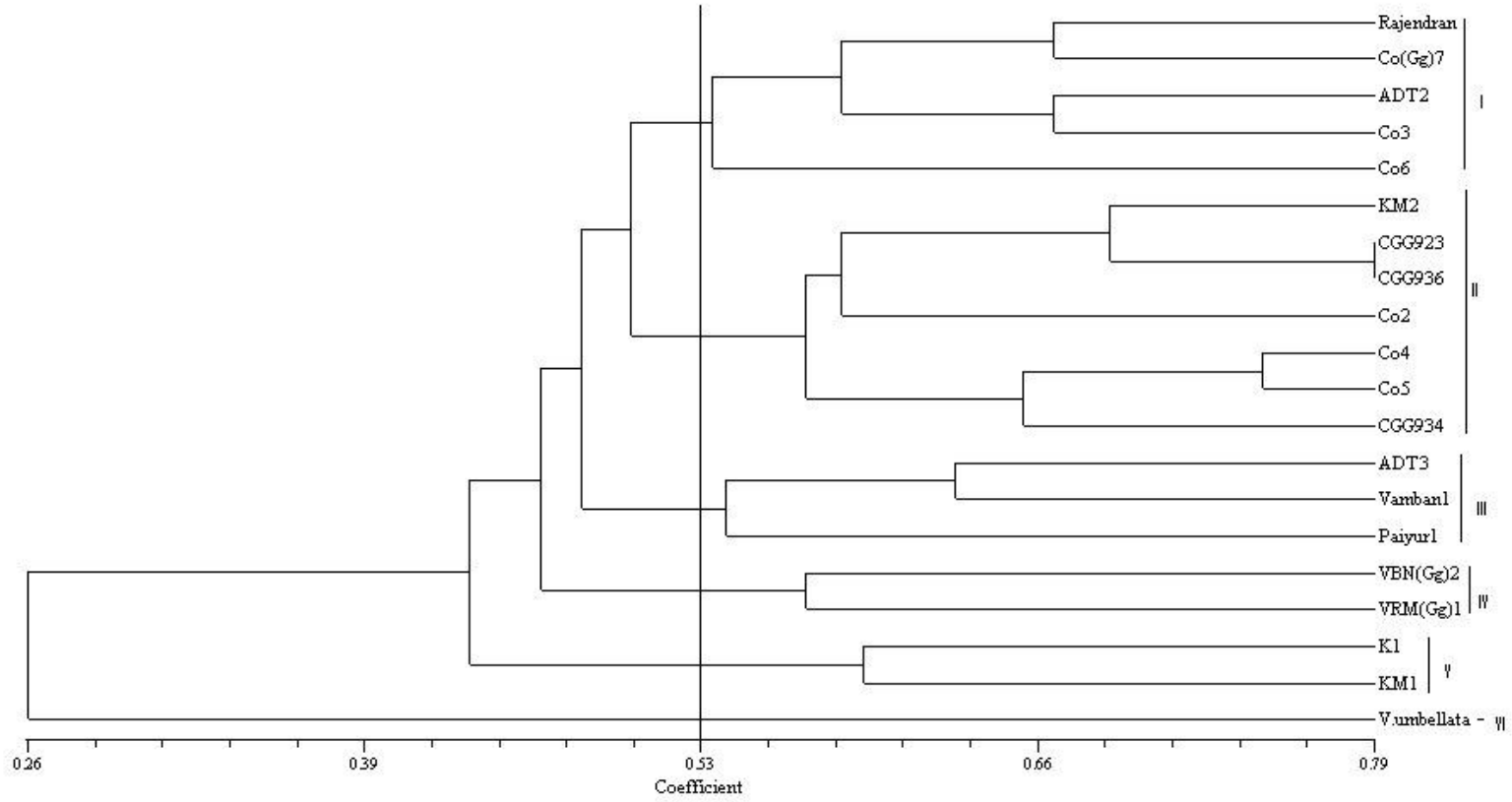

Figure 1. Dendrogram generated using UPGMA analysis showing relationships among greengram genotypes obtained by morphological and SSR data.

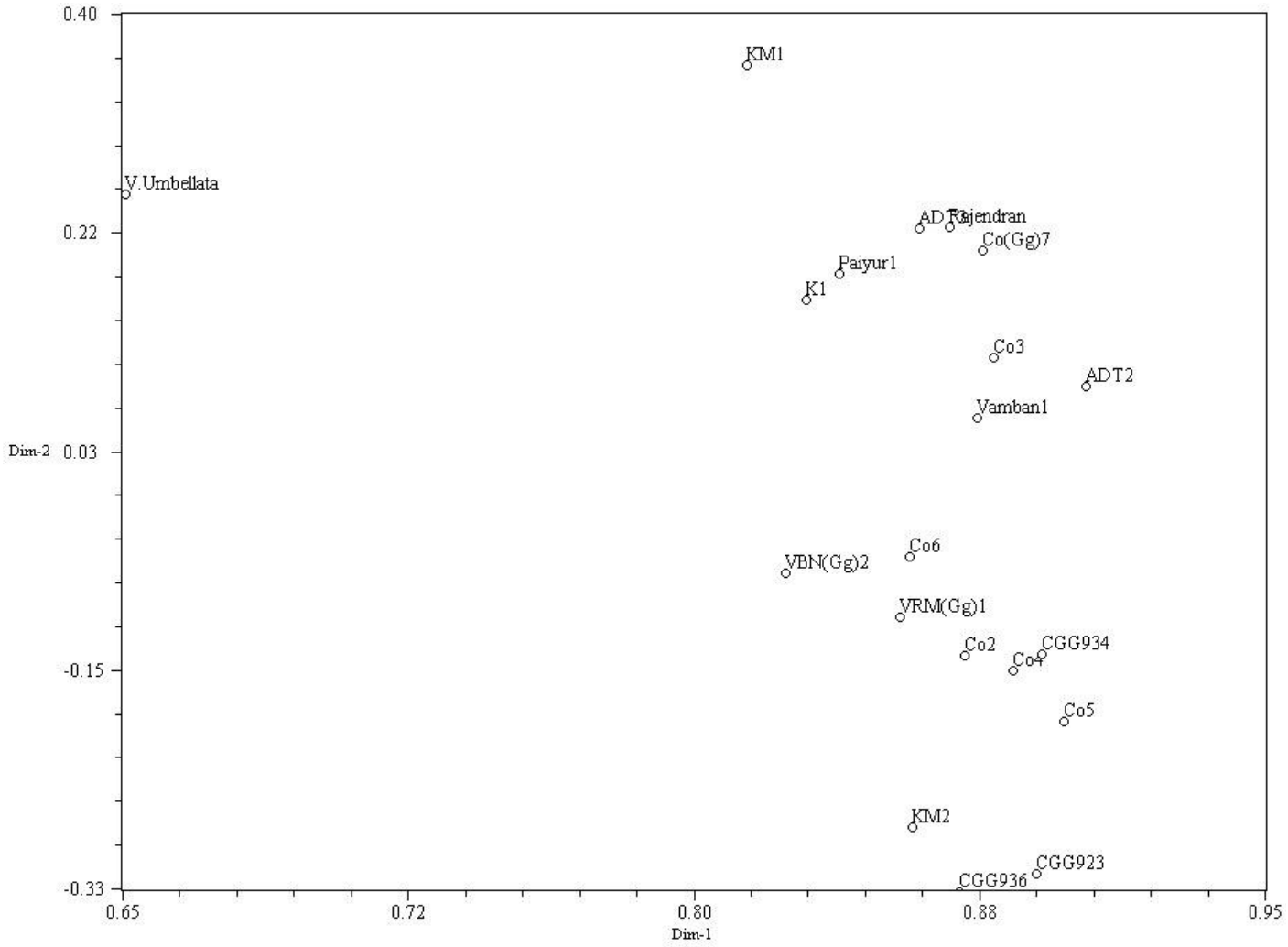

Figure 2. Principal component analysis of 20 greengram genotypes using morphological and SSR data.

relationship in legume species (Blair et al., 2006; Sangiri et al., 2007; Gupta and Gopalakrishna, 2009; Souframanien and Gopalakrishna, 2009; Gwag et al., 2010). The potential for integrating morphological and microsatellite markers into plant improvement is enormous.

In the present investigation morphological and SSR markers were used for diversity analysis among 20 genotypes. Eighteen morphological traits generated 50 alleles. Among the traits studied, seed colour generated the maximum number of alleles (five). Corolla colour, terminal leaflet shape and mature pod colour generated four alleles per trait. Of the 16 SSR primers, which showed amplification in this study, only 14 primers gave polymorphism. A total of 41 alleles were produced. This polymorphism could be attributed to mutations in both the SSR region and the flanking regions contributing to the variation in allele size and number among different genotypes studied. Among the SSR primers, primers derived from adzukibean were found to produce the maximum number of alleles (Table 3). The cross amplification of adzuki bean SSR was studied in *V. radiata* (Sangiri et al., 2007) and in *Vigna mungo* (Chaitieng et al., 2006; Gupta et al., 2008; Gupta and Gopalakrishna, 2009; Souframanien and Gopalakrishna, 2009). Successful amplification of common bean and cowpea SSRs was observed among greengram genotypes studied.

The ability to use the same microsatellite primers in different plant species depends on the extent of sequence conservation in the primer binding sites flanking the microsatellite loci and the stability of the sequence during evolution (Decroocq et al., 2003). Microsatellite primer pairs used in the current study originated from greengram. Adzuki bean, common bean and cowpea and 16 microsatellite primer pairs were able to amplify DNA across all the 20 greengram genotypes studied. This indicates the conservation of

microsatellite sequences among the species during evolution.

Among the SSR markers, marker originated from adzukibean showed the maximum polymorphism among the genotypes studied. Previously, Sangiri et al. (2007) obtained high level of polymorphism in wild and cultivated greengram germplasm using adzuki bean microsatellite markers. Chaitieng et al. (2006) had observed that about 67% of adzuki bean macrosatellite markers gave amplification blackgram and about 26% were polymorphic between cultivated and wild blackgram genotypes. The transferability of microsatellite markers conserved between the species also serves as a valuable tool for comparative mapping studies (Gupta et al., 2008).

Cluster analysis based on morphological and microsatellite data grouped the 20 greengram genotypes into six main clusters. In PCA analysis, genotypes were resolved into five different groups and results were well congruent with the cluster analysis. The long duration variety KM1 grouped separately in PCA. Genotype *V. umbellata* has the lowest similarity and was placed separately from rest of the genotypes in both the dendrogram and the PCA as expected (Figures 1 and 2). This wild species is well known to harbour many novel alleles that can be utilized in plant breeding.

The results indicate that microsatellite markers derived from other legume species namely: adzuki bean, common bean and cowpea could be effectively used for molecular studies in greengram, in which availability of SSRs is fewer. Microsatellite and morphological markers were highly effective in estimating the genetic diversity and genotype identification in greengram. The results in this study show that sufficient variability exists in the elite genotypes of greengram, which were developed and commercialized over a period of 40 years and would be useful in the selection of suitable parents for breeding purposes and genetic mapping studies.

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