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Characterization of Mungbean (*Vigna radiata* L. Wilczek) Varieties using Morphological and Molecular Descriptors

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A B S T R A C T

Five mungbean varieties released by Punjab Agricultural University (PAU911, SML668, ML818, ML613 and SML832) were characterized using morphological and molecular markers. Phenotypically these varieties showed variation for growth habit, leaf and flower characters, pod colour, position and length, plant height, seed coat lusture and seed size during different growth stages of the crop. Plant morphology characters being polygenic in nature are liable to be influenced by the environment. Hence there is a need to use alternate descriptors which are rapid, accurate and less affected by environment. Therefore, in the present study, simple sequence repeats (SSR) markers were also used for determining genetic diversity analysis and supplementing morphological data. Five mungbean varieties were analysed using 44 SSR molecular markers, covering the whole genome of greengram. Out of 44 primers screened primers RG6, RG7, RG8, RG11, RG14, RG15 and MBM00101exhibited distinct banding pattern. Markers RG6 and RG7 differentiate the variety ML818 and SML832. Marker RG11 distinguished ML818 and ML613. Similarly, primers RG8, RG11, RG14 and RG15 showed distinctive banding pattern among the SML832 and ML613. Primer MBM001101 indicated polymorphism among PAU 911 and SML668. The UPGMA dendrogram based on similarity co-efficient showed maximum genetic difference between ML613 and ML818 and maximum similarity between ML818 and SML668.

Introduction

Mungbean (*Vigna radiate* L. Wilczek) or green gram is an important legume crop. It is a great source of proteins, vitamins, and minerals, particularly in South Asia. It is a self-pollinated crop having 2n = 2x = 22 chromosomes with a genome size of 579 Mb/1C. Its capacity to restore soil fertility through nitrogen fixation makes it a valuable crop in various cropping systems, particularly wheat-rice. The Productivity of mungbean is very low *i.e.* only around 500 kg per ha. The low productivity can be attributed to narrow genetic base and lack of suitable genotypes for different cropping situations (Dikshit *et al*., (2009). Being major producer of mungbean, India has developed a large number of commercial cultivars. These varieties are characterised by high degree of homogeneity. The traditional method of variety characterisation is the field sown; grow out test which involves examination of plants from vegetative stage to maturity. This is time consuming since it covers the entire duration of the crop which may extend up to

100 days. Moreover, plant morphology characters being polygenic in nature are liable to be influenced by the environment. Hence there is a need to use alternate descriptors which are rapid, accurate and less affected by environment. Several molecular marker types are available and each has their advantages and disadvantages (Manivannam *et al.,* 2016). The molecular marker based seed purity assay could be a better alternative and is receiving more attention as these are not influenced by genotype and environment interactions, making DNA barcoding as the most straight forward method for cultivar identification (Naresh *et al.,* 2009). A few PCR-based DNA markers, including random-amplified polymorphic DNA (RAPD), sequence-tagged microsatellite site (STMS), single nucleotide polymorphism (SNP), and simple sequence repeat (SSR) has been developed in mungbean (Van *et al.,* 2013). Among them SSR markers or microsatellites (Litt *et al.,* 1989), have been widely used for investigating the genetic relatedness and diversity in plant population and cultivars (Bornet *et al.,* 2006). SSR markers are important owing to their co-dominant inheritance, relative abundance, high reproducibility, polymorphism, and simplicity of genotyping (Tautz *et al.,* 1984; Varshney *et al.,* 2005). Present studies were therefore undertaken to evaluate morphological and molecular characterisation for their genetic diversity.

Materials and Methods

The experimental material consisted of breeder seed of five mungbean (*Vigna radiata* L. Wilczek) varieties released by Punjab Agricultural University, Lidhiana *viz*.PAU911, SML668, ML818, ML613 and SML832. These were planted during March 2014 and 2015 at research farm of Pulses Section of Department of Plant Breeding and Genetics, PAU Ludhiana. For morphological characterisation, all varieties were planted in four rows (5 meters Long) at 45 cm distance in three replications. The plant to plant distance was kept at 15 cm. The observations on morphological characters were recorded during different growth stages. The anthocyanin pigmentation at cotyledonary stage was observed at unfolded stage (5 days after sowing). Similarly, characters like growth habit, plant habit, stem colour, leaf colour, vein colour, leaf size, petiole colour and stem pubescence observed at 50% flowering stage. For the assessment of colour characteristics, the latest Royal Horticultural Society (RHS) colour chart was used. While characters like plant height, premature pod colour were observed when pods were fully developed. Pod colour at maturity, pod curvature, pod position and mature pod length were observed at maturity stage of the crop. Seed characters *i.e.* seed colour, seed coat lusture, seed shape, seed size were observed after harvest. All morphological observations were conducted as per DUS testing guidelines by Protection of Plant Varieties & Farmers' Rights Authority (2007). For molecular analysis whole seedling of each genotype, after removing the seed coat from 7 to 8 days old seedlings, were used for DNA extraction. Genomic DNA was isolated using the CTAB method (Saghai-Maroof *et al.,* 1984) and DNA concentration was estimated using DNA nanodrop. These estimates were confirmed by staining DNA with ethidium bromide after electrophoresis on 0.8 per cent agarose gel at 100V for 1h in TAE buffer (0.4 M Tris-acetate, 0.001 M EDTA, pH 8.0). Ethidium bromide-stained gel was visualized and documented with the help of Gel Documentation System. PCR conditions were standardized using varying concentrations of primers and template DNA. After standardization, the reaction mixture was prepared in 20 µL volume containing 40 ng of template DNA, 1X final concentration of *Taq* buffer B, 25 mM $MgCl_2$, 5.0 µM of each

forward and reverse primer, 2.5 mM of deoxynucleotide triphosphates (dNTPs) and 0.03 U of Taq, DNA polymerase (5 U μL–1) and run on a thermo cycler (mastercycler PCR System; Eppendorf). The PCR conditions used for amplification of SSRs consisted of initial denaturation at 94°C for 45 s, followed by 38 cycles, each consisting of denaturation at 94°C for 30 s, annealing at 50-60°C for 1 min, and elongation at 72°C for 45 s, with a final extension at 72°C for 10 min. The PCR amplified products were resolved on 3.0% agarose gel electrophoresis in 0.5% TAE buffer at a constant power supply with known concentration of DNA ladder (50 ng μL^{-1}; Thermo Scientific) as a molecular weight marker. Ethidium bromide-stained gels were visualized and photographed by using the Gel Documentation System. SSR alleles were scored for the lower molecular weight and higher molecular weight of the SSR marker bands. The amplified products for SSR analysis was scored visually based on molecular size of the band for each marker. Each fragment was treated as a unit and only clear and unambiguous bands were scored. The similarity co- efficients were used to construct a dendrogram for determining relationship using unweighted pair group method with arithmetic average (UPGMA).

Results and Discussion

Morphological characterization

Anthocyanin colouration recorded at seedling stage was present in all the varieties and hence indicated no variation. Other characters *viz.* plant habit, stem colour, stem pubescence, petiole colour, premature pod colour, pod pubescence, pod curvature, seed colour and seed shape, exhibited similar characteristics among all the varieties. Phenotypic data is presented in table 1, for growth habit, leaf colour, leaf size, flower colour and vein colour was recorded at 50% flowering stage. It indicated that *cv.* SML613 had semi erect growth habit while other varieties had erect growth habit. SML 668 and SML832 exhibited dark green leaf colour and PAU911, ML818 and ML613 showed green leaf colour. Likewise, for leaf size three types of observations were registered *i.e.* ML818 and ML613 had medium size leaf, while SM668 and SML832 were observed with broad leaf and PAU911 had large leaf size. Flower colour was yellow in variety PAU911, while remaining all were distinct with respect to light yellow colour. The vein colour was greenish purple in PAU911 and light purple in all other varieties. Assessment of characteristics like plant height and pod position was recorded at fully developed green pod stage. Varieties like SML668 and SML832 remained dwarf (<50cm) while varieties PAU911, ML818 and ML613 were medium tall (50-70cm). The pod position was observed above the canopy in PAU911, SML 668 and SML832 and below the canopy in ML613 and ML818. Characteristics like pod colour at maturity and pod length were recorded during maturity. Varieties PAU911 and ML668 exhibited dark brown podcolour while varieties SML 668 and SMl832 had blackish brown pod colour and ML613 produced brown cloured pods. Likewise, pod length was observed to be medium long in ML613 and SML832 and long in PAU911, SML668 and ML818. The characteristics of seed coat luster and seed size was analyzed at mature seed stage. The seed coat luster for variety SML668 was medium shining while for all others it was shining. On the other hand seed size grouped the varieties into three categories, *i.e.* medium for PAU911, SML818, ML613 and bold size in variety SML668. Third type was of medium bold in the variety SML832. Though these morphological characters could distinguish some varieties in both the seasons but there were few plants in the group of 50 plants under observation which indicated some deviations at certain points of growth stages.

Table.1 Morphological characters in different mungbean varieties

Characters	Stage of observation	Varieties				
		PAU911	**SML668**	**ML818**	**ML613**	**SML832**
Hypocotyl: Anthocyanin colouration	Cotyledons unfolded	Present	Present	Present	Present	Present
Plant type	50% flowering	Erect	Erect	Erect	Semi-erect	Erect
Plant growth habit	50% flowering	Determinate	Determinate	Determinate	Determinate	Determinate
Stem colour	50% flowering	Green	Green	Green	Green	Green
Stem pubescence	50% flowering	Present	Present	Present	Present	Present
Leaf colour	50% flowering	Green	Dark Green	Green	Green	Dark Green
Vein colour	50% flowering	Greenish purple	Light purple	Light purple	Light purple	Light purple
Petiole colour	50% flowering	Greenish purple	Greenish purple	Greenish purple	Greenish purple	Greenish purple
Leaf size	50% flowering	Large	Broad	Medium	Medium	Broad
Flower colour	50% flowering	Yellow	Light yellow	Light yellow	Light yellow	Light yellow
Premature pod colour	Fully developed green pod	Green	Green	Green	Green	Green
Pod pubescence	Fully developed green pod	Present	Present	Present	Present	Present
Pod position	Fully developed green pod	Above canopy	Above canopy	Below canopy	Below canopy	Above canopy
Plant height	50% flowering	Medium tall	Dwarf	Medium tall	Medium tall	Dwarf
Days to Maturity	Harvest maturity	75	60	80	85	61
Pod colour at maturity	Harvest maturity	Dark brown	Blackish brown	Dark brown	Black	Blackish brown
Pod curvature	Harvest maturity	Slightly curved at beak	Slightly curved at beak	Slightly curved at beak	Slightly curved at beak	Slightly curved at beak
Mature pod length (< 8 cm)	Harvest maturity	Long	Long	Long	Medium long	Medium long
Seed colour	Mature seeds	Green	Green	Green	Green	Green
Seed coat lusture	Mature seeds	Shining	Medium shining	Shining	Shining	Shining
Seed shape	Mature seeds	Oblong/Oval	Oblong	Oblong	Oblong	Oblong
Seed size (3.5 g/100 seed)	Mature seeds	Medium	Bold	Medium	Medium	Medium Bold
No. of grains per pod	Maturity	9-11	10-11	10-1	11-12	10
Yield qlts/ha	Maturity	4.9	4.5	4.9	4.3	4.6

Table.2 Markers used for genetic diversity in mungbean

Sr.No.	Marker	Sr.No.	Marker
1	RG1	23	MBM00037
2	RG2	24	VRO274
3	RG3	25	VRO244
4	RG4	26	MBM0002
5	RG5	27	VR0487
6	RG6	28	VRO413
7	RG7	29	VRO393
8	RG8	30	VRO390
9	RG9	31	MBM00012
10	RG10	32	MBM00023
11	RG11	33	MBM00036
12	RG12	34	MBM00042
13	RG13	35	MBM00011
14	RG14	36	MBM00101
15	RG15	37	MBM00082
16	VR0248	38	MBM00097
17	VR0135	39	MBM00060
18	VR0133	40	MBM00049
19	VRO73	41	MBM00171
20	VRO95	42	MBM00173
21	VRO375	43	MBM00106
22	VR0361	44	MBM00155

Table.3 Maker analysis indicating polymorphism in different varieties of mungbean

Sr. No.	Polymorphic marker	Variety
1.	RG6	ML818,SML832
2.	RG7	ML818,SML832
3.	RG8	SML832,ML613
4.	RG11	SML832,ML613 and ML818, ML613
5.	RG14	SML832,ML613
6.	RG15	SML832,ML613
7.	MBM00101	PAU911,SML668

Fig.1-4 PCR amplification in 5 different varieties of mungbean by using 44SSR primers

Figure 1

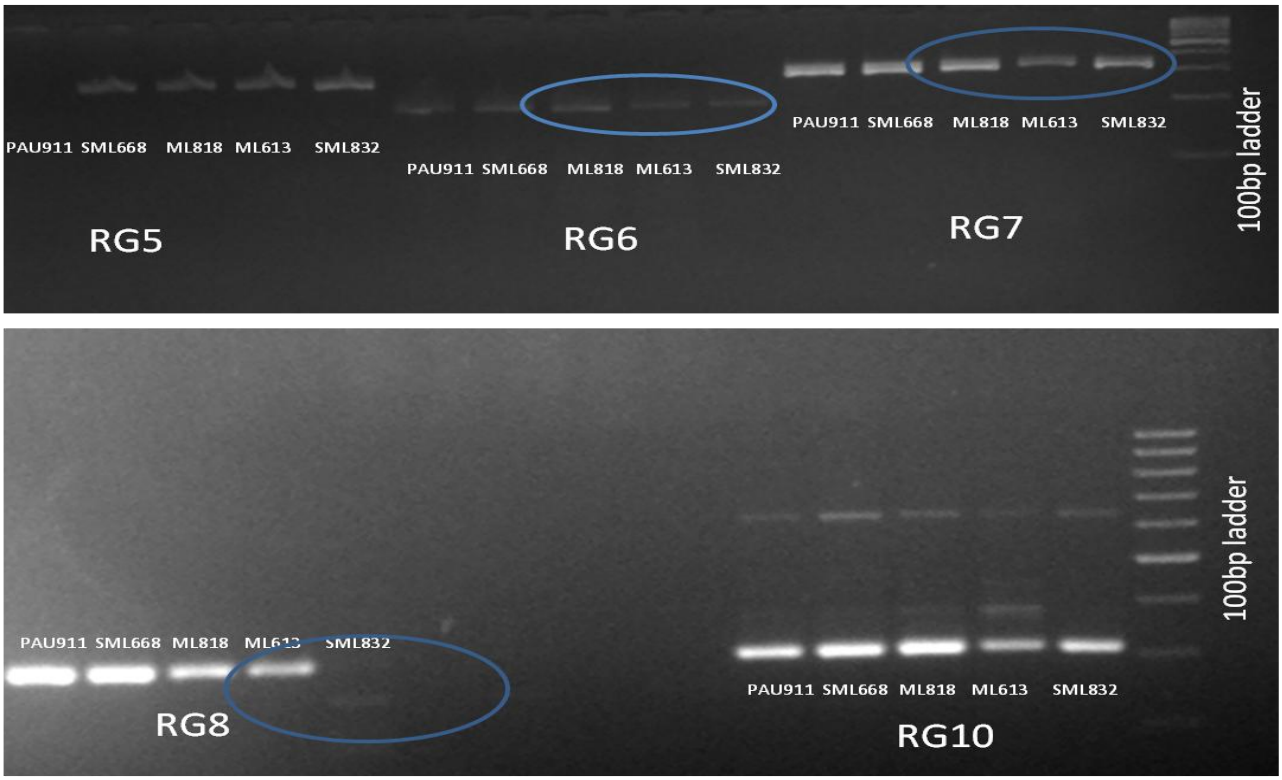


Figure 2

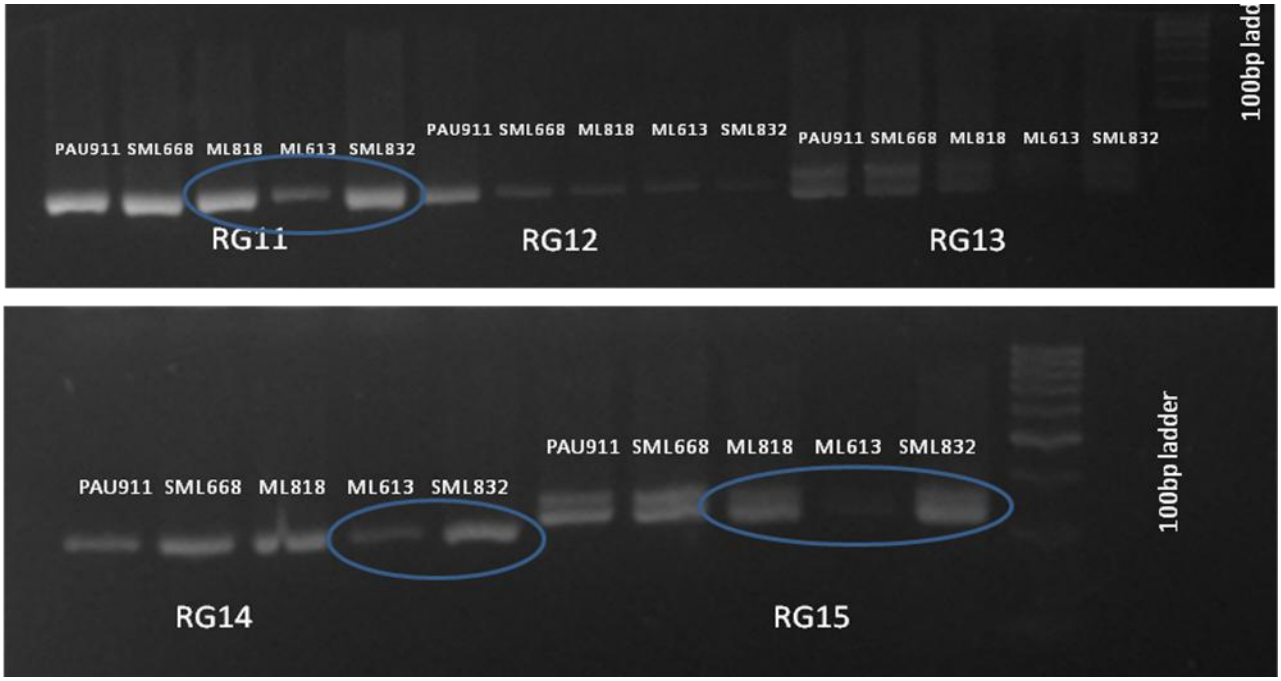


Figure 3

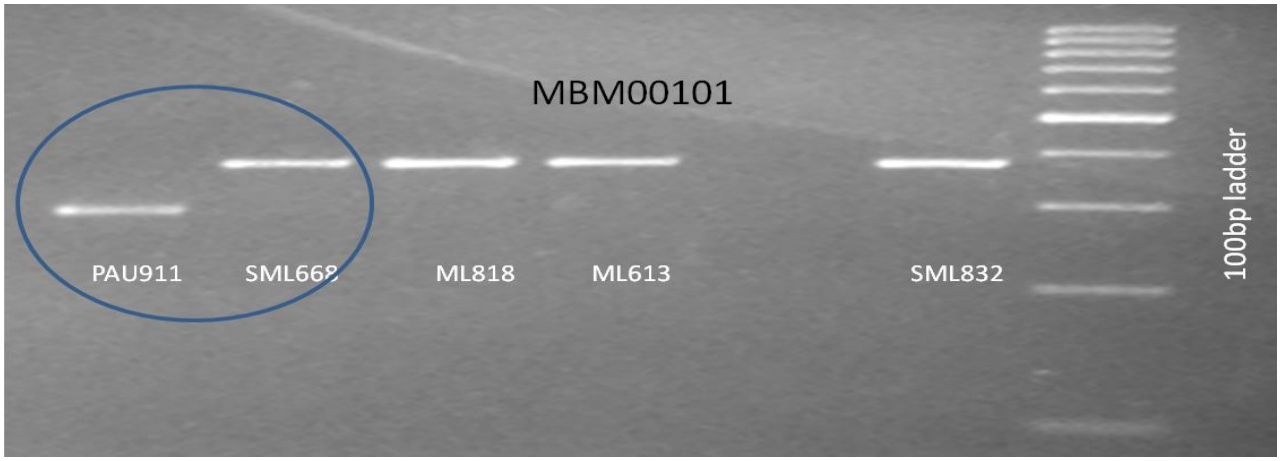


Figure 4

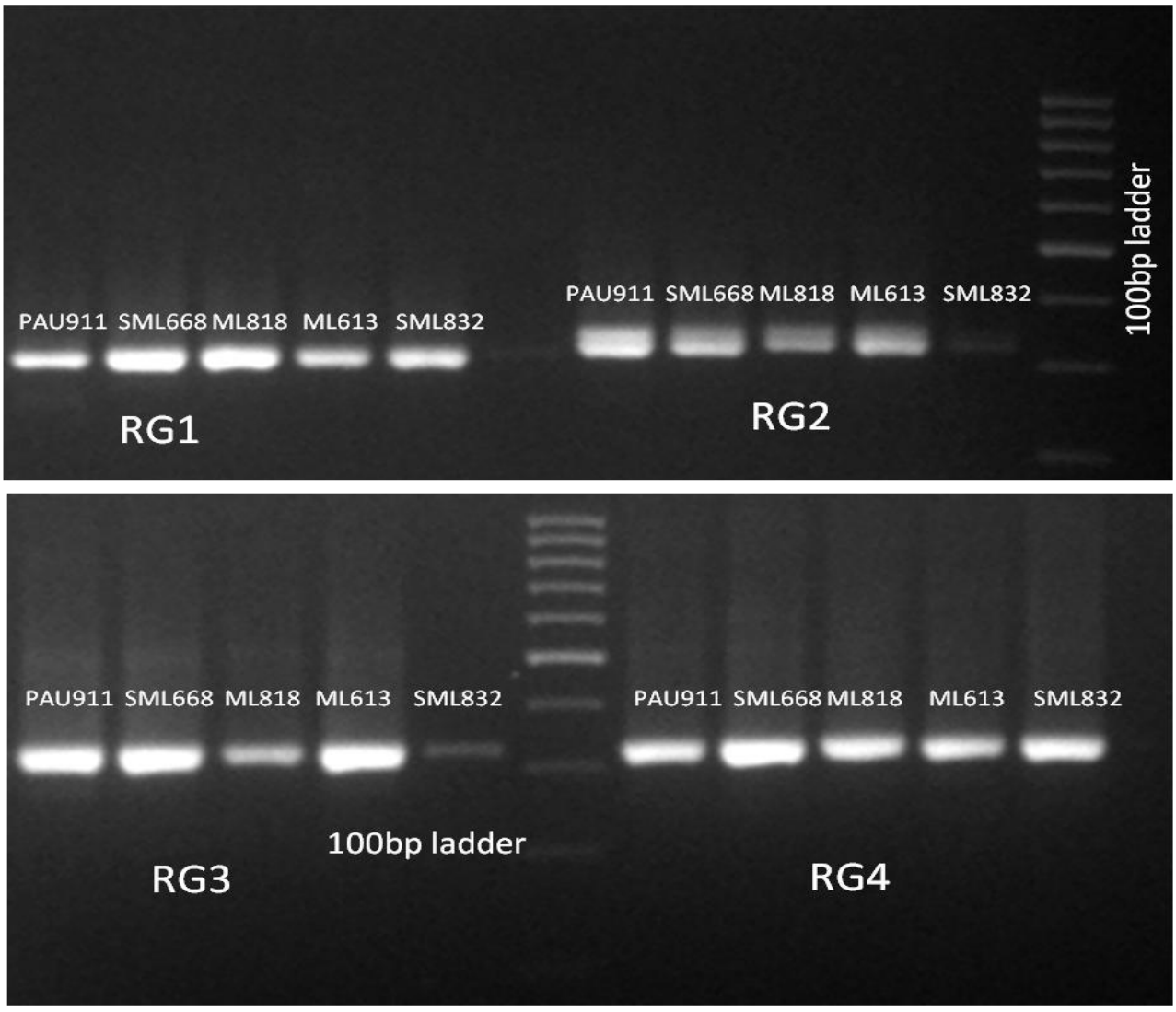
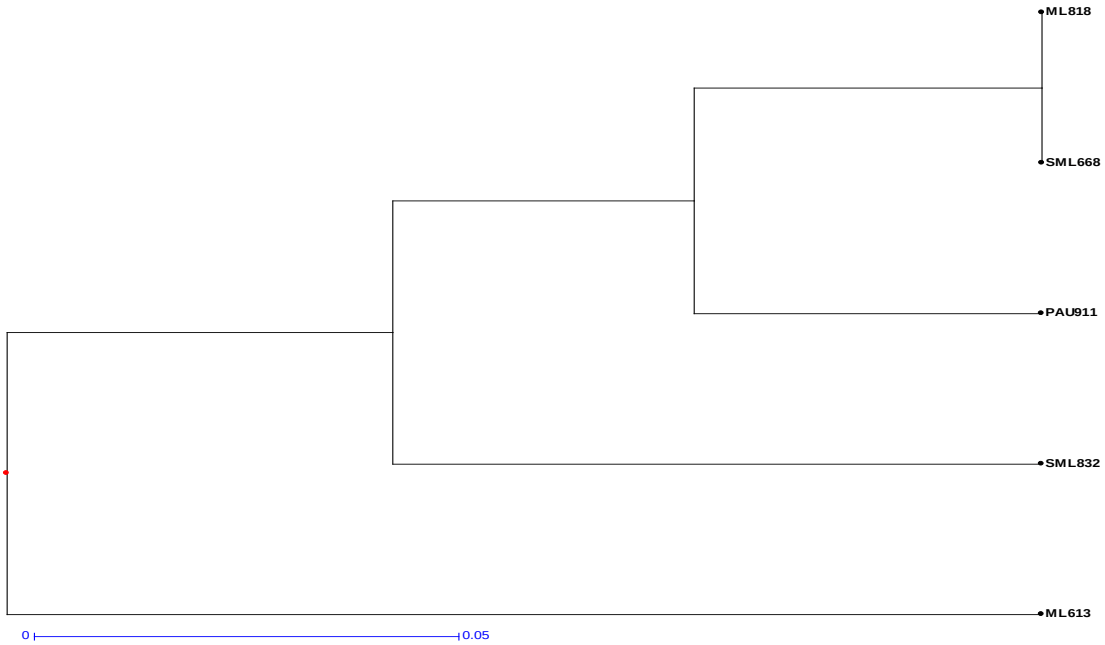


Fig.5 Dendrogram of mungbean varieties constructed using UPGMA based on 44 SSR markers



Such observations emphasis the need of molecular markers. Therefore for relevant distinctiveness among the varieties, SSR marker analysis became more significant.

Molecular characterization

For varietal characterization, molecular marker analysis was done in five mungbean varieties. For this 44 SSRs were applied, out of which 27 primers gave clear and consistent amplification profiles with all the five mungbean varieties shown in table 2. Among these primers, 7 showed polymorphism, while 20 were monomorphic presented in table 3. Remaining primers failed to show amplification, revealing no bands (null allele) or failed to amplify for more than one variety. The similarity co- efficient values were used to construct the UPGMA (Unweighted Pair Group Method on Arithmetic Average) presented in figure 5. The dendrogram depicting the genetic relationships generated using DARwin 5.0 Programme classified the varieties into three distinct groups, with*cv.*ML818 andSML668 showing highest genetic similarity, whereas, ML613 was highly distinct from other varieties. Likewise, PAU911 and SML832 also showed the genetic variation. Grouping of the genotypes as revealed by the clustering analysis was congruent with the pedigree and breeding history of the varieties. DNA marker analysis makes it possible for researchers to pinpoint specific fingerprint and accurately identify a particular genotype. It has been found that DNA fingerprinting profile for the genotype ML818 and SML832 by the primers the RG6 and RG7, for SML832 and ML613 by RG8, RG11, RG14 and RG15 positions is unique and presented in figures 1-3. Likewise in figure 3, genotypes PAU911 and SML668 with MBM00101 primer showed the unique bands. Similarly RG11 also resulted into differentiation between ML818 and ML613 shown in figure 2. SML668, PAU911 and SML832 showed similarity as most of the markers showed the monomorphic DNA

barcoding presented in figure 4. Thus from the above investigation, it may be concluded that molecular analysis revealed substantial polymorphism in these mungbean varieties. The technique may be used to obtain reasonably precise information on genetic relationship among mungbean genotypes. Such information may be useful for selecting the diverse parents and monitoring the genetic diversity periodically in the breeder's working collection of mungbean. It is very useful for variety testing and genetic purity. Extensive molecular diversity analysis was done by using different molecular markers using RAPD markers, Sunitha *et al.,* (2012) using 24 ISSR markers and Wang *et al.,* (2012) using 15 SSR markers reported polymorphism in different mungbean genotypes used in their study. Genetic diversity analysis using PCR based markers (RAPD, SSR and ISSR) revealed that, higher marker indices were obtained for ISSR markers, which proved to be the most efficient marker system in terms of average heterozygosity values (Akanksha *et al.,* 2014). The genetic diversity within released cultivars of Indian mungbean has been rather sporadic and commonly used parents in breeding programs have missed out from these studies. It has been described that diversity of Indian mungbean cultivars is narrow (Gupta *et al.,* 2013) also reviewed genetic linkage maps, comparative genome mapping, and gene/quantitative trait loci (QTLs) mapping for agronomically important traits of this crop. Lakhanpaul *et al.,* (2000) studied the diversity among 32 mungbean cultivars from India using RAPD markers. Singh (2003) used ISSR marker to access genetic diversity to mungbean genotypes. Bhat *et al.,* (2005) also used AFLP marker system to assess the genetic diversity and relationship among the cultivars. The number of genome wide polymorphic SSR markers is limited for greengram (Somta *et al.,* 2008 and Tangphatsornruang *et al.,* 2009). Hence, the diversity profiles of these genotypes could be exploited for future varietal identification.

It was thus concluded that morphologically flower and vein colour distinguish the PAU911 variety from rest of the varieties under study. But SSR markers currently used could precisely distinguish all the five varieties from each other. Therefore these SSR markers can further be exploited to differentiate other mungbean genotypes.

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