International Journal of Genetics
ISSN: 0975-2862 & E-ISSN: 0975-9158, Volume 11, Issue 5, 2019, pp.-587-593.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000226

Research Article

GENETIC DIVERSITY & TRANSFERABILITY OF CHICKPEA - SEQUENCE TAGGED MICROSATELLITE SITES (STMS) PRIMERS TO *VIGNA* & *PHASEOLUS* SPECIES

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Received: May 01, 2019; Revised: May 14, 2019; Accepted: May 15, 2019; Published: May 30, 2019

Abstract: Eight chickpea STMS primers were used to amplify 31 genotypes of eight *Vigna* & one *Phaseolus* species collected from GBPUA & T, Pantnagar, PCPGR, Pantnagar & Banaras Hindu University Banaras. Number of alleles per locus ranged from 3 for STMS primer TA59, which varied in size from 100 to 500 bp. Amplification of genomic DNA of thirty one genotypes, yielded 10 fragments, with an overall mean of 0.8 alleles per locus of which 9 were polymorphic while the remaining one was monomorphic. The eight STMS primers show an overall of 95.833% polymorphism. The PIC value ranged from 0.54 for locus TA59 to 0.375 for locus TA27 with a mean of 0.29. The cluster analysis using the UPGMA method displayed three clusters with 2, 5 & 24 genotypes respectively. The locus-wise total gene diversity (HT) ranged from 0.177 at TA59 to 0.499 at TA27, with an overall mean of 0.354 & the major allele frequency ranged from 0.516 for TA27 to 0.8925 for TA59, with an overall mean of 0.740. Five out of the eight chickpea STMS primer-pairs were >70% successfully transferable across the *Vigna* & *Phaseolus* species. The highest level of successful amplifications with a single primer TA110 was 83.87%. The present study indicates that the locus-specificity, co-dominant nature & transferability of STMS markers permit the fast & high throughout fingerprinting of genotypes from one genus to other to estimate their genetic diversity.

Keywords: Diversity, STMS, *Vigna*, alleles, UPGMA

Citation: Bhareti P.,*et al*., (2019) Genetic Diversity & Transferability of Chickpea - Sequence Tagged Microsatellite Sites (STMS) Primers to *Vigna* & *Phaseolus* Species. International Journal of Genetics, ISSN: 0975-2862 & E-ISSN: 0975-9158, Volume 11, Issue 5, pp.- 587-593.

Introduction

Pulses occupied the main position in Indian agriculture. Pulses are rich in protein and also constitute the main source of essential amino acids. Grain legumes are among the most important crops in many countries & provide one-third of human dietary protein. Most of the economically important grain legumes belong to the genus *Vigna*. Despite systematic & continuous efforts through traditional breeding methods, substantial yields in these grain legumes could not be achieved. Mungbean (*Vigna radiata* (L.) Wilczek,) also known as greengram is widely cultivated in three different seasons in India *i.e*., *kharif* (rainy), *rabi* (winter) & *zaid* (spring & summer). A native to India-Burma area, it is grown in South, Southeast & East Asian countries. The major consumption of mungbean is as food legume. It is having short growing period & easily fits in different cropping systems of India [1]. The average productivity of the crop of less than 400 kg/ha is lower than that of other pulses [2]. India is the largest producer of mungbean, with 1.2 mt harvested from about 3.5 mha. In terms of production area, mungbean is the third most valuable legume crop in India after chickpea (7.37 mha) & pigeon pea (3.63 mha) [3]. The low productivity of this crop can be attributed to narrow genetic base & lack of suitable plant types for different cropping situations. *Vigna radiata* var. sublobata is considered to be the putative progenitor of mungbean. Mungbean is a relatively drought hardy crop, but less tolerant to water logging. Heavy rainfall during the pod ripening stage can cause premature sprouting, leading to poor quality seeds [4]. Mungbean remains largely a village crop, with relatively few reports of studies at the molecular level on its molecular diversity & useful traits [5]. Bruchid infestation & MYMV are two major productivity constraints for mungbean production in South & South-East Asia. Development of molecular markers can facilitate the breeding to incorporate & pyramid resistance gene into commercial cultivars. Blackgram (*Vigna mungo* (L.) Hepper) is the fourth important pulse crop in India, covering an area of about 3mha but productivity is only 1.2mt. It is cultivated in different seasons in India *i.e*., in *kharif* (rainy season) as a mixture with cereals, pigeonpea etc. and in *rabi* & *zaid* (spring & summer) as pure culture. Blackgram is also grown in Pakistan, Bangladesh, Sri Lanka & Burma. Like mungbean, it is also originated from Indian subcontinent [6, 7]. It is believed that black gram was domesticated in northern South Asia from *V. mungo* var. silvestris [8]. Several high yielding and disease resistant cultivars developed through routine breeding approaches have been released. In spite of this, production, area & productivity have remained virtually stagnant over the last few years in India. Molecular breeding is expected to play a far greater role in blackgram breeding. Molecular markers are used for the study of genetic diversity in blackgram [9, 10]. However, there are no reports of the development of a genetic linkage map for blackgram. The rice bean {*Vigna umbellata* (Thunb.)} is native to South-Eastern Asia. The common name of ricebean is Japanese rice bean, climbing mountain bean, Mambi bean (Gaimoong in Bengali), Sutari (in Hindi) and Shiltong (in Nepali). Rice bean is cultivated to limited extent to India, Burma, Malaysia, Japan, Java, China, Fiji, Mauritius, Korea and Philippines [11, 12]. Rice bean is used both as a green manure, food & fodder. In India, it is sown in June-July & harvested in October-November as a *kharif* crop. It is also cultivated on aman paddy in summer months & also as catch crop in between *kharif* & *rabi* season [13]. Common bean (*Phaseolus vulgaris* (L.)) is an important legume crop used as green pod vegetable (known by several names as snap bean, string bean, garden bean, fresh bean) or dry seeds (known as dry beans). The dry seed type varieties are called as "Rajmash" in India. The common bean originated in the new world, principally Central & South America [14]. Now it is widely cultivated in the tropics, subtropics & temperate regions. In terms of both production & consumption, Brazil is far ahead of other countries [15]. Bean is mainly used in two forms, namely, dry bean & snap bean. Dry bean accounts for 57% production of the world food legume & nearly 80% of dry bean production occurs in the developing countries [16]. Cultivated common bean originated from its wild relatives.

Two principal domestication centres have been identified (Middle America and Andean highlands) by Harlan [17]. In India, common bean is mostly used as a dry seed legume. However, slight improvements are needed in seed yield, greater nitrogen fixation, resistance to *Fusarium* wilt, bean common mosaic virus and tolerance to pod borer. The conventional plant breeding approaches need support of molecular assisted selection to accelerate breeding in a precise manner. Common bean breeders have traditionally developed new cultivars by selection & adaptation of superior lines. In common bean, genetic diversity has been evaluated in the past using both morphological & molecular markers [18].

Molecular markers are very important for characterizing and determine genetic diversity among common beans [19]. RFLPs were principally used as framework markers to develop molecular linkage maps in common beans [20, 21]. RAPDs have been extensively used, not only to develop linkage maps, but also to characterize genetic diversity [22, 23]. AFLPs have also proven useful for developing low-density linkage maps [24]. Cowpea (*Vigna unguiculata* (L.)) also called as southernpea and blackeyed pea, is well adapted to the tropics and is an important legume crop, ranking fifth in the world as a source of fibres and plant protein. Cowpea is originated in Africa. In India it is known by different names. The name lobiya is probably of Greek origin, derived from the word lobos, meaning a pod. The crop is grown on about 12.5 mha in warm to hot regions of the world. About two thirds of the production & more than three fourths of the area of production is in Africa. The major cowpea growing countries are Nigeria, Burkina Faso, Ghana, Kenya, Uganda, Malawi, Tanzania (all in Africa) and India, Sri Lanka, Burma, Bangladesh, Philippines, Indonesia & Thailand etc. In India the estimated area is about 5, 00,000 ha. Cowpea mainly used as a dry seeds, fodder, green pod, green manures & cover crops. The wild subspecies *Vigna unguiculata* ssp. *dekindtiana* is the progenitor of modern cowpea than the other wild subspecies, mensensis. Except hills it is grown in all parts of India. It is frequently grown with other crops like maize, sorghum, millets & cassava but sometimes grown as a pure crop. In India mainly ssp. *unguiculata* & ssp. *cylindrica* types have predominance while yard long bean (ssp. *sesquipedalis*) forms are grown on small scale for green vegetables. In India, cowpea does not appear to be a major pulse crop or vegetable crop. As a fodder crop, it plays an important role. DNA markers should be more effective for developing in linkage map for cowpea. Genetic engineering has considerable potential for making possible unique types of progress in cowpea breeding. For cowpea, it will not replace traditional breeding methods but could provide genes from other species that confer resistance to abiotic and biotic knowledge of germplasm diversity using molecular markers provides a significant impact on the improvement of crop plants. Molecular markers have been used to identify duplicate accessions, genetic relationships, & population structures, & to determine how variation is distributed between individuals accessions, & races. Marker assisted selection for those traits which are difficult to screen phenotypically has become an important tool for breeding programs. Molecular markers are now widely used to track loci & genome regions in many important crops including legume crops & several improved varieties have been developed using molecular markers in recent times [25].

The evolution of legumes was already studied using molecular tools. Among these tools are used as various kinds of DNA markers, which demonstrated their potential to reveal genome evolution of related taxa & to analyse genome structure. One type of DNA markers, so called sequence-tagged microsatellite sites (STMS) markers, has been recognized as ideal for genotype identification [26]. Microsatellites are frequently & randomly used in eukaryotic genomes & comes under highly informative markers. These locus specific microsatellite markers (STMS) are amplified from known sequences that flank microsatellite arrays. They are produced using clones obtained from microsatellite enriched cDNA libraries. These clones are screened for the presence of preferably long & perfect arrays, which allow suitable flanking primers to be designed. Although the production of STMS markers is labour intensive & costly, but constitutes high reproducibility, locus specificity & codominance than other molecular markers. STMS markers also shown good transferability across closely related genera [27, 28] & consequently are fast becoming an important tool for molecular diversity analysis & genome mapping in plants. Microsatellites can be isolated directly from genomic DNA libraries, cDNA libraries, libraries enriched for specific microsatellites [29]. It has recently been shown that STMS markers are three times more efficient as compared to dominant markers for intraspecific analysis & are equally efficient as other dominant markers in assessing interspecific variations [30]. Markers generated by sequence tagged microsatellite site (STMS) primer pairs are particularly suitable for genome analysis. STMS markers are PCR-based, may provide single-locus detection and may be co-dominantly inherited. The variability of microsatellites is exploited by a PCR-based technique that uses microsatellite flanking sequences as primers to amplify the microsatellites in between. The resulting locus-specific amplification products often exhibit considerable length differences due to variations in the number of tandem repeats within the microsatellite. Molecular breeding research is still lagging behind in *Vigna* species compared to other grain legumes such as common bean & soybean despite their importance. There is an urgent need to develop a large number of molecular markers in *Vigna* species, which can be used by breeders in various molecular breeding strategies to increase the breeding efficiency.

Materials and Methods

Plant material

The experimental material comprised of 31 genotypes of *Vigna* & *Phaseolus* species including Mungbean, Blackgram, Rice bean, Common bean, Cowpea & wild relatives of blackgram & mungbean. The seeds were obtained from GBPUA & T Pantnagar, PCPGR Pantnagar & Banaras Hindu University, Banaras, India. For DNA extraction plants were sown in the N. E. Borlaug Crop Research Centre, G. B. Pant University of Agriculture & Technology, Pantnagar during *kharif* season of 2012-13 & molecular marker analysis was carried out at Pantnagar Centre for Plant Genetic Resources (PCPGR) Laboratory, G. B. Pant University of Agriculture & Technology, Pantnagar.

DNA extraction

Genomic DNA was extracted from young leaves of each seedling separately (one seedling per genotype) following the standard CTAB method as described by Doyle & Doyle (1987) [31]. About 0.5 grams of fresh leaves of each seedling were ground separately with the help of liquid N_2 to fine powder using pre-chilled pestle and mortar. Then homogenized in 3 ml of pre-warmed (60ºC) CTAB extraction buffer (2% (w/v) CTAB, 100 mM Tris-Cl, 1.4 M NaCl, 20 mM EDTA and 0.2% beta mercaptoethanol) and incubated at 65°C in water bath for 1 hour. During incubation microtubes were gently mixed after every 15 minutes by inverting the tubes several times. After 1 hour, tubes were cooled to room temperature and 3 ml of chloroform isoamyl alcohol (24:1 v/v) was added in each microtube under fume hood. The microtubes were gently mixed for 5 minutes by inverting and spun for 10 minutes at 10,000 rpm at 4°C. A clear interface was seen in-between two liquid phases. The upper aqueous phase was transferred carefully into clean microtubes. Then the DNA was precipitated with a cold solution of isopropanol (double of the recovered volume of aqueous phase). The microtubes were gently inverted to be sure mixing is complete before incubating at -20°C for overnight. After, microtubes were spun for 10 minutes at 4°C at 10,000 rpm & the supernatant was removed. The solution was poured without disturbing the DNA pellet at the bottom of the tubes. The pellet DNA was washed with 70% ethanol by mixing gently for 20 minutes. Then, the microtubes were spun for 5 minutes at 4°C. The supernatant was removed & the pellet DNA was air dried at room temperature by leaving microtubes open. The pellet was dissolved in 200 µl of TE buffer (pH 8.0). The solution was treated with 5 µl RNase (10 mg/ml), incubated at 37°C for 1 hour. The tubes were centrifuged at 10,000 rpm for 5 minutes & the top layer of DNA was removed. To this, sodium acetate (1/10 vol., pH = 4.8) & chilled absolute ethanol was added. The contents were mixed & kept at –20°C for 30 min. Finally, the pellet was washed with 70 percent ethanol, dried & dissolved in l00 µl of TE buffer. Quality of DNA was checked on a 1 % agarose gel & its concentration was determined by taking the absorbance on UV-Vis spectrophotometer. The optical density was measured at 260 & 280 nm. A portion of the DNA was diluted in molecular grade water to make 50 ng concentrations & stored at -20°C.

Table-1 List of various varieties of *Vigna* & *Phaseolus* species used in the study

Crop	Type	Variety	Crop	Type	Variety
Black gram		Uttara	Cowpea	Grain	Pant lobia 2
		WBU 108			Pant lobia 3
		Pant U 19		Fodder	UPC 625
		Pant U 30			UPC 628
		Pant U 35			Pant lobia 1
		Pant U 31	Common bean		Pant bean 2
		Pant U 40	Mung bean		Ganga 8
		HUM 12			MH -2-15
		HUM 16			ML 818
Blackgram	Wild (Black gram)	VBN 1X			IPM 02-3
					Pant moong 2
		VNB 4X			Pant moong 3
		ADT 3X			Pant moong 4
Mungbean	Wild (Mung bean)	*Vigna*			Pant moong 5
		trilobata			Pant moong 6
Ricebean		PRR 2008-2			
		PCPGR8404			
		PRR 2007-2			

Table-2 Characteristics of STMS primers used in this study

Primer code	Forward sequence	Reverse sequence	Total no of nucleotides
TA96	TGTTTTGGAGAAGAGTGATTC	TGTGCATGCAAATTCTTACT	21-20=41
TA59	ATCTAAAGAGAAATCAAAATTGTCGAA	GCAAATGTGAAGCATGTATAGATAAAG	27-27=54
CS27A	AGCTGGTCGCGGGTCAGAGGAAGA	AGTGGTCGCGATGGGGCCATGGTG	24-24=48
TA37	ACTTACATGAATTATCTTTCTTGGTCC	CGTATTCAAATAATCTTTCATCAGTCA	27-27=54
TR59	AAAAGGAACCTCAAGTGACA	GAAAATGAGGGAGTGAGATG	20-20=40
TA27	GATAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG	26-27=53
TA110	ACACTATAGGTATAGGCATTTAGGCAA	TTCTTTATAAATATCAGACCGGAAAGA	27-27=57
TA194	TTTTTGGCTTATTAGACTGACTT	TTGCCATAAAATACAAAATCC	23-21=44

Table-3 STMS Primer amplification of thirty one genotypes of *Vigna* & *Phaseolus* species

SN	Primer code	Number of loci amplified	Number of monomorphic loci	Number of polymorphic loci	Polymorphism (%)	Major allele frequency	Gene diversity (HT)	Polymorphic information content (PIC)	Range of amplified loci (bp)
1	TA96	1	0	1	100	0.645	0.457	0.352	100
2	TA59	3	1	2	66.67	0.892	0.177	0.154	100-500
3	CS27A	1	0	1	100	0.774	0.349	0.289	100
4	TA37	1	0	1	100	0.645	0.457	0.353	100
5	TR 59	1	0	1	100	0.806	0.312	0.263	100
6	TA 27	1	0	1	100	0.516	0.499	0.375	100
7	TA110	1	0	1	100	0.838	0.27	0.234	100
8	TA194	1	0	1	100	0.806	0.312	0.263	100

PCR amplification & STMS identification

A set of eight polymorphic STMS primers of chickpea were used to characterize thirty one genotypes of *Vigna* & *Phaseolus* species [Table-1]. These markers have been previously demonstrated to be useful by in distinguishing chickpea genotypes. PCR amplifications were performed using eight chickpea STMS primers-pairs [Table-2]. Reactions were carried out in 25 µl volume containing 2.5 µl of PCR buffer, 50 ng/µl of genomic DNA, 2.5 mM each of dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 25 mM $MgCl_2$, 0.01% gelatin, 1µl of forward & reverse primers & 5U/µl Taq DNA polymerase (Bangalore Genei, Bangalore, India). DNA amplification was performed using an Eppendorf Master Cycler gradient (Eppendorf Netheler-Hinz, Hamburg, Germany). Conditions for PCR reactions were: one cycle at 94°C for 5 minutes, 44 cycles at 94°C for 1 minute, 50°C for 2 minutes, 72°C for 2 minutes & a final cycle of 72°C for 7 minutes. The amplified loci were separated by 2% agarose gel in 0.5×TBE buffer (pH 8.0) at 80 V. The gel was stained with 0.8 µg/ml ethidium bromide solution & 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.

The amplification products were viewed under UV light & photographs were saved for the experimental evaluation. The bands were scored for the presence or absence by binary coding *i.e.*, assigning a value of 1 for presence & 0 for absence in a lane. Molecular weight of amplified DNA fragments was determined by the DNA ladder marker used in the first well of the gel.

Estimation of genetic diversity

Data were analyzed with Numerical Taxonomy Multivariate Analysis System software package (NTSYSpc version 2.11W) [32]. A similarity matrix (Jaccard's similarity coefficients) was constructed based on SIMQUAL programme [33].

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

Where, N_{AB} is the number of bands shared by samples, NA represents amplified fragments in sample N_A & N_B represents fragments in sample B. Similarity matrices based on these indices were calculated.

Clustering was done by UPGMA using SHAN module of NTSYSpc to determine genetic relationships among the accessions studied. Polymorphism information content (PIC) values, allele frequency & gene diversity were calculated at each locus using the POWERMARKER software [34]. PIC provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles that are expressed but also the relative frequencies of those alleles. Allele number is counted as the band number with different size of each STMS locus.

Table-4 Similarity matrix of 31 genotypes (*Vigna* & *Phaseolus*) using Jaccard's coefficient calculated from STMS banding pattern using 8 STMS markers

Genotypes	Uttara	WBU 108	Pant U 19	Pant U 30	Pant U35	Pant U31	Pant U 40	HUM 12	HUM 16	VBN 1X	VNB 4X	ADT 3X	*Vigna trilobata*	PRR 2007-2	PRR 2008-2
Uttara	1														
WBU 108	1	1													
Pant U 19	1	1	1												
Pant U 30	1	1	1	1											
Pant U 35	0.9	0.9	0.9	0.9	1										
Pant U 31	0.9	0.9	0.9	0.9	1	1									
Pant U 40	0.9	0.9	0.9	0.9	1	1	1								
HUM 12	0.8	0.8	0.8	0.8	0.9	0.9	0.9	1							
HUM 16	1	1	1	1	0.9	0.9	0.9	0.8	1						
VBN 1X	0.9	0.9	0.9	0.9	1	1	1	0.9	0.9	1					
VNB 4X	1	1	1	1	0.9	0.9	0.9	0.8	1	0.9	1				
ADT 3X	1	1	1	1	0.9	0.9	0.9	0.8	1	0.9	1	1			
Vigna trilobata	1	1	1	1	0.9	0.9	0.9	0.8	1	0.9	1	1	1		
PRR 2007-2	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.7	0.9	0.8	0.9	0.9	0.9	1	
PRR 2008-2	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.7	0.9	0.8	0.9	0.9	0.9	1	1
PCPGR 8404	0.8	0.8	0.8	0.8	0.9	0.9	0.9	0.8	0.8	0.9	0.8	0.8	0.8	0.7	0.7
Pant Bean	1	1	1	1	0.9	0.9	0.9	0.8	1	0.9	1	1	1	0.9	0.9
Pant lobia1	0.9	0.9	0.9	0.9	1	1	1	0.9	0.9	1	0.9	0.9	0.9	0.8	0.8
Pant lobia2	0.7	0.7	0.7	0.7	0.8	0.8	0.8	0.7	0.7	0.8	0.7	0.7	0.7	0.6	0.6
Pant lobia3	0.9	0.9	0.9	0.9	1	1	1	0.9	0.9	1	0.9	0.9	0.9	0.8	0.8
UPC 625	0.7	0.7	0.7	0.7	0.8	0.8	0.8	0.7	0.7	0.8	0.7	0.7	0.7	0.6	0.6
UPC 628	0.7	0.7	0.7	0.7	0.8	0.8	0.8	0.9	0.7	0.8	0.7	0.7	0.7	0.6	0.6
Ganga 8	0.8	0.8	0.8	0.8	0.7	0.7	0.7	0.8	0.8	0.7	0.8	0.8	0.8	0.9	0.9
MH -2-15	0.8	0.8	0.8	0.8	0.7	0.7	0.7	0.8	0.8	0.7	0.8	0.8	0.8	0.9	0.9
ML 818	0.3	0.3	0.3	0.3	0.4	0.4	0.4	0.5	0.3	0.4	0.3	0.3	0.3	0.4	0.4
IPM02-3	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.6	0.6	0.5	0.6	0.6	0.6	0.7	0.7
Pant moong 2	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.4	0.2	0.3	0.2	0.2	0.2	0.3	0.3
Pant moong 3	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.6	0.6	0.5	0.6	0.6	0.6	0.7	0.7
Pant moong 4	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.4	0.2	0.3	0.2	0.2	0.2	0.3	0.3
Pant moong 5	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.4	0.2	0.3	0.2	0.2	0.2	0.3	0.3
Pant moong 6	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.4	0.2	0.3	0.2	0.2	0.2	0.3	0.3

	PCPGR 8404	Pant Bean 2	Pant lobia 1	Pant lobia 2	Pant lobia 3	UPC 625	UPC 628	Ganga 8	MH -2-15	ML 818	IPM02-3	Pant moong 2	Pant moong 3	Pant moong 4	Pant moong 5	Pant moong 6
PCPGR 8404	1															
Pant Bean 2	0.8	0.1														
Pant lobia 1	0.9	0.9	1													
Pant lobia 2	0.9	0.7	0.8	1												
Pant lobia 3	0.9	0.9	1	0.8	1											
UPC 625	0.7	0.7	0.8	0.6	0.8	1										
UPC 628	0.7	0.7	0.8	0.8	0.8	0.6	1									
Ganga 8	0.6	0.8	0.7	0.5	0.7	0.5	0.7	1								
MH -2-15	0.6	0.8	0.7	0.5	0.7	0.5	0.7	1	1							
ML 818	0.3	0.3	0.4	0.4	0.4	0.4	0.6	0.5	0.5	1						

Table-5 Transferability of chickpea STMS primers across *Vigna* & *Phaseolus* species

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	Percent transferability
Chickpea STMS primers																																
TA96	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	+	+										64.52
TA59	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+								77.42
CS27A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+		+	+			+		+				77.42
TA37	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+											64.52
TR59	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+		+	+	+		+		+				77.42
TA27	+	+	+						+		+	+	+	+	+		+						+	+		+		+				48.39
TA110	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+		+				83.87
TA194	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							80.65

Results and Discussions

All the eight chickpea STMS primer pair used in the study gave good amplification & transferability in the expected band size across the different genera. Primers were used for analysis on the basis of easily scoreable bands & showed an overall of 95.833% polymorphism. The number of bands amplified, as resolved in 2% agarose gel by each of the primers ranged from 1 to 3, a total of 10 alleles with an average of 0.8 alleles per locus were amplified in 31 genotypes. Maximum of three alleles were observed for primer TA59 & it exhibited 66.67% polymorphism. Most of the primers TA96, CS27A, TA37, TR59, TA27, TA110 & TA194 amplified only single allele & showed 100% polymorphism. The PIC value for eight STMS markers obtained in the present study varied from 0.154 for TA59 to 0.375 for TA27, with an average PIC value for the 8 markers to be 0.29. Lower PIC values results from the closely related genotypes & the vice versa. The major allele frequency for eight STMS markers obtained in the present study, varied from 0.516 for TA27 to 0.8925 for TA59, with an average major allele frequency for the 8 markers to be 0.740. The value of gene diversity for eight STMS markers varied from 0.177 for TA59 to 0.499 for TA27, with an average value of gene diversity for the 8 markers to be 0.354 [Table-3]. Similarity, coefficient between any two genotypes estimated based on DNA amplification by STMS primers varied from 0.20 to 1.00 [Table-4]. The vast range of similarity coefficient between any two genotypes indicated the presence of wide genetic variability among the genetic stocks studied. The lowest similarity coefficient 20% was observed for different pairs of genotypes viz., Pant moong 2 & Uttara, Pant moong 4 & Uttara, Pant moong 5 & Uttara, Pant moong 6 & Uttara, Pant moong 2 & WBU 108, Pant moong 4 & WBU 108, Pant moong 5 & WBU 108, Pant moong 6 & WBU 108, Pant moong 2 & Pant moong 19, Pant moong 4 & Pant moong 19, Pant moong 5 & Pant moong 19, Pant moong 6 & Pant moong 19, Pant moong 2 & Pant moong 30, Pant moong 4 & Pant moong 30, Pant moong 5 & Pant moong 30, Pant moong 6 & Pant moong 30, Pant moong 2 & HUM 16, Pant moong 4 & HUM 16, Pant moong 5 & HUM 16, Pant moong 6 & HUM 16, Pant moong 2 & VNB 4X, Pant moong 4 & VNB 4X, Pant moong 5 & VNB 4X, Pant moong 6 & VNB 4X, Pant moong 2 & ADT 3X, Pant moong 4 & ADT 3X, Pant moong 5 & ADT 3X,

Pant moong 6 & ADT 3X, Pant moong 2 & *Vigna trilobata*, Pant moong 4 & *Vigna trilobata*, Pant moong 5 & *Vigna trilobata*, Pant moong 6 & *Vigna trilobata*, Pant moong 2 & PCPGR 8404, Pant moong 4 & PCPGR 8404, Pant moong 5 & PCPGR 8404, Pant moong 6 & PCPGR 8404, Pant moong 2 & Pant bean-2, Pant moong 4 & Pant bean-2, Pant moong 5 & Pant bean-2 & between Pant moong 6 & Pant bean-2. Highest similarity coefficient was observed between WBU 108 & Uttara, Pant U 19 & Uttara, Pant U 30 & Uttara, HUM 16 & Uttara, VNB 4X & Uttara, ADT 3X & Uttara, *Vigna trilobata* & Uttara, Pant bean-2 & Uttara, Pant U 19 & WBU 108, Pant U 30 & Pant U 19, VNB 4X & Pant U 19, ADT 3X & Pant U 19, *Vigna trilobata* & Pant U 19, Pant bean-2 & Pant U 19, HUM 16 & Pant U 30, VBN 1X & Pant U 30, ADT 3X & Pant U 30, *Vigna trilobata* & Pant U 30, Pant bean-2 & Pant U 30, Pant U 31 & Pant U 35, Pant U 40 & Pant U 35, VBN 1X & Pant U 35, Pant lobia 1 & Pant U 35, Pant lobia 3 & Pant U 35, Pant U 40 & Pant U 31, VBN 1X & Pant U 31, Pant lobia 1 & Pant U 31, Pant lobia 3 & Pant U 31, VBN 1X & Pant U 40, Pant lobia 1 & Pant U 40, Pant lobia 3 & Pant U 40, VNB 4X & HUM 16, ADT 3X & HUM 16, *Vigna trilobata* & HUM 16, Pant bean-2 & HUM 16. Pant lobia 1 & VBN 1X, Pant lobia 3 & VBN 1X. ADT 3X & VNB 4X, *Vigna trilobata* & VNB 4X, Pant bean-2 & VNB 4X, *Vigna trilobata* & ADT 3X, Pant bean-2 & ADT 3X, Pant bean-2 & *Vigna trilobata*, PRR 2008-2 & PRR 2007-2, Pant lobia 3 & Pant lobia 1, MH-2-15 & Ganga 8, Pant moong 3 & IPM 02-3, Pant moong 4 & Pant moong 2, Pant moong 5 & Pant moong 2, Pant moong 6 & Pant moong 2, Pant moong 5 & Pant moong 4, Pant moong 6 & Pant moong 4 & between Pant moong 6 & Pant moong 5.

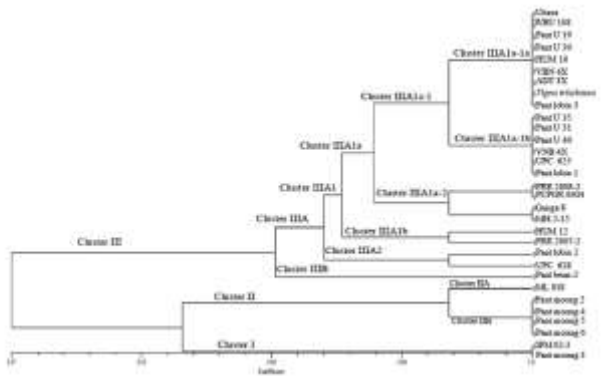

Fig-1 Dendrogram of *Vigna* & *Phaseolus* species based on UPGMA cluster analysis

A dendrogram derived from UPGMA cluster analysis based on the dice similarity coefficient matrix for 31 genotypes was constructed [Fig-1]. Based on the dendrogram from UPGMA, the 31 genotypes were grouped into three clusters with 2, 5 & 24 genotypes in I, II & III, respectively & separated at 57.8% (cluster I & II) & 37% (cluster III from cluster I & II) similarity level. All the wild genotypes form a separate cluster III. The genotype *Vigna mungo*, *Vigna umbellata, Vigna unguiculata* & *Phaseolus vulgaris*, separated from *Vigna radiata* & formed a distinct cluster (Cluster III). The smaller cluster, cluster I having only two genotypes IPM 02-3 & Pant moong 3 & cluster II comprised five genotypes Pant moong 2, Pant moong 4, Pant moong 5, Pant moong 6 & ML 818, belonging to the genus *radiata*. Cluster II comprised of two subclusters, IIA & IIB at 90.4 % similarity level. The major cluster, cluster III comprised twenty-four genotypes. Among these, twenty-three genotypes formed sub cluster IIIA at 0.69% similarity level & one genotype Pant bean 2 separated from other genotypes of cluster III & came in subcluster IIIB. Cluster IIIA was again subdivided into two clusters, IIIA1 & IIIA2 at 75.4 % similarity level. Cluster IIIA1 comprised of twenty one genotypes, namely, Uttara, WBU 108, Pant U 19, Pant U 30, HUM 16, VNB 4X, ADT 3X, *Vigna trilobata*, Pant lobia 3, Pant U 35, Pant U 31, Pant U 40, VBN 1X, UPC 625, Pant lobia 1, PRR 2008-2, Ganga 8, PCPGR 8404, MH-2-15, HUM 12 & PRR 2007-2. Cluster IIIA2 comprised only two genotypes, namely, Pant lobia 2 & UPC 628. Cluster IIIA1 was again subdivided into subcluster IIIA1a & IIIA1b, which were similar to each other at 77.8 % similarity level. Cluster IIIA1b comprised only two genotypes, HUM 12 & PRR 2007-2. Whereas. cluster IIIA1a comprised nineteen genotypes & further subdivided into subcluster IIIA1a-1 & IIIA1a-2 (80.8% similar to each other). Cluster IIIA1a-2 comprised of four genotypes, namely, PRR 2008-2, PCPGR 8404, Ganga 8 & MH-2-15. Cluster IIIA1a-1 was further subdivided into IIIA1a-1a & IIIA1a-1b with fifteen genotypes (90.4 % similarity level). Cluster IIIA1a-1a comprised of nine genotypes, namely, Uttara, WBU 108, Pant U 19, Pant U 30, HUM 16, VNB 4X, ADT 3X, *Vigna trilobata*, & Pant lobia 3. Whereas, IIIA1a-1b comprised of six genotypes, namely, Pant U 35, Pant U 31, Pant U 40, VNB 4X, UPC 625 & Pant lobia 1. Measures of intra- & inter-species transferability of chickpea STMS primers to the other species tested are shown in [Table-5]. Five out of the eight chickpea STMS primer-pairs were >70% successfully transferable across the *Vigna* & *Phaseolus* species.

Moreover, chickpea STMS primer pair, TA110 was most successful, amplifying 26 out of the total 31 genotypes (83.87%). Primer pair TA194 generated 80.65% transferability across the genera & amplifying 25 out of the total 31 genotypes, whereas, primers TA59, CS27A & TR59 amplifying 24 out of the total 31 genotypes with transferability across the genera was 77.42%. Furthermore, 3 chickpea STMS primer-pairs produced between 45-65% positive amplifications. The potential transferability of highly polymorphic STMS primers to amplify across species, expressed as the total mean percentage of successful amplification, was 71.77 % for chickpea STMS primers. The individual mean percentage of successful transferability of chickpea STMS primers across blackgram (9), wild blackgram (3), wild mungbean (1), rice bean (3), cowpea (grain+fodder=5), common bean (1) & mungbean (9) was 91.67%, 95.83%, 100%, 79.17%, 87.5%, 75% & 34.72% respectively.

Molecular markers are projected to have a major impact on the genetic improvement of a wide range of species [35]. Molecular markers can detect differences in DNA sequences & are less ambiguous than phenotypic markers. The polymerase chain reaction described by Mullis *et al*., (1986) [36] is an important tool in molecular biology. In recent years, many DNA markers have been developed & have become powerful tools for detecting genetic diversity. Molecular markers offer particular promise in enabling breeders to target desirable physiological traits, especially those that are time consuming to measure &/or are subject to large genotype X environment interaction. Molecular markers also allow comparative mapping analysis, which detects genomic conservation (synteny) in closely & distantly related species & can therefore help transfer information & resources from well studied to less studied genomes [37]. Molecular markers can help elucidate the genetic basis of desirable traits & the nature of changes through selection [38] & have been used for construction of genetic linkage maps in many crop species. In turn, these maps have helped the detection of quantitative trait loci (QTLs) associated with many physiological traits like seed weight in mungbean [39]. Our PIC values were about average & could be explained by *Vigna* & *Phaseolus* genetic diversity. Metais *et al*., (2002) [19] published a range of 0.12–0.72 for PIC values, with an average of 0.44 when evaluating 15 polymorphic SSRs in 45 different bean lines, belonging to nine different quality types. Cluster analysis allowed common bean germplasm Pant bean 2 to be divided into a separate group. Molecular markers have been used to assist common bean breeding programs in various ways.

The clustering of most improved cultivars of mungbean in cluster I & II & blackgram in cluster III suggests that the genetic basis of commercial blackgram & mungbean cultivars in India is narrow relative to genetic variation in wild relatives. Further, only weak associations were observed between subgroup membership & geographic origin of the genotypes [Fig-1]. This is likely the result of the improved cultivars from broad geographic areas having common parentage as a result of exchanges of improved materials among breeding programs. Such a narrow genetic base increases vulnerability to widespread pathogen epidemics as well as limiting genetic gain from selection in the future. Hence, it is necessary to import additional sources of genetic variation to ensure long term genetic gain & to reduce susceptibility to pathogen/pest attack. Valuable genes/traits for genetic improvement of elite *Vigna* & *Phaseolus* cultivars no doubt exist among the landraces, wild relatives & in the other subspecies. A backcross hybridization between wild & cultivated genotypes, blackgram X mungbean hybridization & ssp. *unguiculata* & *sesquipedialis* accessions followed by limited backcrossing could be a useful strategy for incorporating useful genetic variation from exotic germplasm, or other suggested strategies for introgression of exotic germplasm [40].

The markers used in this study provide a resource for marker-assisted breeding, in particular the application of marker-assisted backcross or introgression breeding approaches to incorporate additional genetic variation into *Vigna* breeding programs to help ensure genetic gain & introgression of valuable resistance traits.

A set of eight polymorphic STMS markers was selected for dendrogram construction. Cluster analysis of the thirty one genotypes of *Vigna* & *Phaseolus* species yielded a dendrogram comprising three main clusters, each of which corresponded with a different *Vigna* sub-genus. Cluster I comprised only the two *Vigna radiata* genotypes which exhibited generally high similarity coefficients (1.00). Cluster II comprised the five mungbean genotypes & cluster III comprised the twenty four genotypes of *Vigna radiata*, *Vigna umbellaata, Vigna unguiculata, Phaseolus vulgaris* & wild species [Fig-1]. The lowest similarity coefficient 20% confirmed the differentiation of the genotypes at the genetic level. Highest similarity coefficient depicted that these genotypes were more similar genetically. The similarity coefficient between most of the genotypes lies between 70 to 100 percent similarity coefficient that give indication that the genotypes are more or less similar to each other. Phansak *et al*. (2005)[41] also observed a high level of genetic diversity within 15 accessions of yardlong bean using STMS analysis & clustered the accessions into three distinct groups, at a 0.67 coefficient of similarity, with no correlation to geographical origins. Three pairs of accessions appeared to be similar indicating that each pair was of the same accession & most likely originated from the same geographical location. STMS analysis with these primers also revealed a very high level of variation between six *Vigna* species, which clustered into three groups at a 0.5 coefficient of similarity. Group A represented the African *Vigna* species yardlong bean & cowpea, while groups B (mungbean, blackgram & moth bean) & C (rice bean & adzuki bean) represented the Asian *Vigna* species. Pandey *et al*., (2011) [42] recorded average Jaccard's similarity coefficient among all pairs of comparisons was 0.695. This was shown by Yu *et al*., (1999) [43] in common bean as well as they found that AT dinucleotides were more frequent than GA motifs in *Phaseolus vulgaris* L. & *Vigna unguiculata* (L.) Walp. However, they were rarely isolated in our study, probably because (AT)n repeats are palindromic & therefore may have not been efficiently enriched during the capture process & because the library was enriched with CT & GT motifs. The allele range encountered in our study is consistent with other common bean reports. While GA-rich repeats were generally identified as the most frequent type of microsatellites in plants [44]. The relative & absolute frequencies of particular motives can vary considerably & unpredictably among species. Eight chickpea STMS primer-pairs used in the study to amplify mungbean, blackgram, ricebean, common bean & wild relatives genomic DNA, only one single band was generally produced, suggesting that the tested plants are all homozygous at the respective loci. The number of heterozygotes was well below 10%. A similar situation was encountered in other self-pollinating species such as soybean, where no single heterozygous plant was found among 43 investigated genotypes [45], & *Arabidopsis thaliana*, where natural populations were characterized by fixed microsatellite alleles in a homozygous state. Restricted polymorphism & low heterozygosity are expected in self-pollinating species [46]. The microsatellite TA59 detected three alleles in the test set. Extremely high variation was revealed by the TA59 primer pair when tested on a larger set of samples. The numbers of alleles at this locus are in a similar range as reported for the most variable STMS loci in soybean. STMS markers are certainly superior to DNA fingerprint bands because they are inherited more reliable, alleles are clearly defined, & non-parental bands are not apparent. Because of their codominant nature, STMS markers will be easily transferable between populations segregating for different traits. They will therefore complement existing mapping approaches based on RAPDs, microsatellite-primed PCR products, AFLPs & other dominant markers in crosses segregating for, e.g. salt resistance [47]. Our results demonstrate that STMS markers derived from chickpea are able to amplify loci in related genera & produced an overall of 95.83% polymorphism, as shown here for blackgram, ricebean, cowpea, common bean, & confirm that chickpea & these genera are very similar as far as the conservation of microsatellite flanking sequences is concerned. This is consistent with the evolutionary distance between these species. Amplification across genera boundaries is possible when the respective loci are conserved between genera. If a locus is not amplified at all, then either mutations occurred at the primer binding site or the locus is not present at all. However, the amplification of a particular locus in one genome with primers designed for another species depends not only on the evolutionary distance between the two species, but also on the rate of base substitutions. It should be stressed that the amplification of DNA with a defined primer pair does not necessarily imply the identity or similarity of the amplified sequences, unless this is verified by sequencing. Extensive polymorphism of the flanking regions was observed between species as well as among accessions within species as detected by differences in the size & number of the amplification products obtained. For instance, the number of amplification products generated by the chickpea STMS primer-pair, TA59 ranged from zero to three for genotype Pant lobia 1 & UPC 625 of cowpea & Pant Bean 2 for common bean. Differences in number of alleles amplified could be caused either by the production or loss of primer binding sites at similar loci. In the case of the chickpea primer-pairs TA59, a monomorphic 500 kbp fragment was amplified in one genotype, Pant Bean 2 representing *Phaseolus* species.

Variation in the size of bands from different individuals was likely to be due to differences in the number of tandem repeats present between the primer sites [27]. Transferability chickpea derived STMS markers, across blackgram, mungbean, rice bean, cowpea, common bean & wild relatives were confirmed in this study. Chickpea STMS primers successfully amplified across most genotypes used, indicating a very high level of sequence conservation among the flanking regions of these microsatellites regions. This was not surprising, since most of the grain legumes belongs to the same tribe, Phaseoleae. However, the transferability of chickpea STMS primers across mungbean, was low. This indicated that the sequences flanking the microsatellite regions in chickpea were different to that in the other pulses. This may be expected since chickpea is placed in the tribe, Cicereae [48]. Alternatively, the methodology used to identify the flanking sequence of the microsatellites in chickpea may have been more selective for *Cicer*-specific sequences. Whereas the methods used for mungbean sequence identification may have selected sequence regions highly conserved across the pulses. The level of marker transferability across species can be used as an indication of the level of sequence conservation & of genetic relationship between these species. Our results allow two interpretations: first, some primer pairs developed for one genome amplified homologous regions in both genomes. These loci are probably conserved between the two tested genera & good candidates for STMS marker design. Second, primer pairs derived from two different STMS loci that produce reliable amplification products recognize other areas of the target genome, which are flanked by sequences complementary to primer sequences of the two STMS loci mixed.

Application of research: The current investigation will be used to characterize genetic variability & to identify the transferability of chickpea STMS markers in different genotypes of *Vigna* & *Phaseolus* species.

Research Category: Genetics and Plant Breeding

Abbreviations:

UPGMA – Unweighted pair group method with arithmetic mean
CTAB – Cetyl trimethylammonium bromide
EDTA – Ethylenediaminetetraacetic acid
DNA – Dioxyribose nucleic acid
rpm – Revolutions per minute
PCR – Polymerase chain reaction

Acknowledgement / Funding: Authors are thankful to Department of Genetics and Plant Breeding. Govind Ballabh Pant University of Agriculture & Technology, Pantnagar, Uttarakhand 263 145, India

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Research project name or number: PhD Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Govind Ballabh Pant University of Agriculture & Technology, Pantnagar, Uttarakhand, PCPGR Pantnagar & Banaras Hindu University, Banaras, India.

Cultivar / Variety name: Table -1

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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