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Microsatellites markers to study genetic relationships among cowpea (*Vigna unguiculata*) genotypes

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ABSTRACT

Genetic diversity and phylogenetic relationships among 48 cowpea [*Vigna unguiculata* (L.) Walp.] genotypes collected from different places in India were evaluated using simple sequence repeat molecular markers. A set of 40 primer combinations developed from cowpea genomic/expressed sequence tags and evaluated for their ability to detect polymorphisms among the various cowpea genotypes. Eleven primer combinations detected polymorphisms; sixteen primer combinations were monomorphic, with the remaining 13 primer sets failing to yield PCR amplification products. From one to 5 alleles were found among the informative primer combinations. The genetic diversity of the sample varied from 0.02 to 0.39 (mean = 0.205). The polymorphic information content ranged from 0.12 to 0.86 (mean = 0.49). The inter-cluster representatives of distant clusters (based on morphological and molecular markers) would be more useful for choosing the parents in cowpea breeding programmes.

Keywords: Cowpea, Genetic diversity, Germplasm management, Microsatellites, *Vigna unguiculata*

Genetic diversity in cowpea has been extensively studied both in wild and cultivated genotypes using various approaches, viz. allozymes analysis (Panella and Gepts 1992, Pasquet 1993), morphological and physiological parameters (Perrino *et al*. 1993, Ehlers and Hall 1996), seed storage proteins (Fotso *et al.* 1994), chloroplast DNA polymorphism (Vaillancourt and Weeden 1992); random amplified polymorphic DNA (RAPD, Mignouna *et al.* 1998, Diouf and Hilu 2005), restriction fragment length polymorphisms (RFLP; Li *et al*. 2001); amplified fragment length polymorphisms (AFLP; Tosti and Negri 2002, Fang *et al.* 2007), DNA amplification fingerprinting (DAF) (Spencer *et al.* 2000 and Simon *et al.* 2007), inter-simple sequence repeat (Ghalmi *et al*. 2010); simple sequence repeats analysis (SSRs; Li *et al.* 2001, Uma *et al*. 2009, Gupta and Gopalakrishma 2010, Asare *et al.* 2010); sequence tagged microsatellite sites (Choumane *et al.* 2000), and cross species SSRs from *Medicago truncatula* (Sawadogo *et al.* 2010). Among all these approaches, SSRs analysis has been particularly useful and informative since these sequences. SSRs have been exclusively used in genetic mapping, genome analysis, genetic variation studies among various legume crops (Li *et al*. 2001, Hong *et al*. 2010, Cieslarova *et al.* 2010).

Microsatellites due to their versatile nature, are one of the molecular markers recommended in a system of distinctness, uniformity and stability as approved by the International Union for the protection of new varieties of plants (UPOV-BMT: BMT/36/10, 2002). Gen-Bank offers a great opportunity for the identification and development of SSR markers by reducing time and cost for developing microsatellite-enriched libraries (Gupta and Gopalakrishma 2010) and with the exception of the study of Diouf and Hilu (2005), who examined 11 cowpea varieties and inbred lines using SSR markers, the diversity and relatedness of cowpea germplasm in India is poorly understood.

The aim of this study is to assess the potential previously analyzed SSRs marker in determining genetic diversity and relationships among cowpea varieties and cultivars found in India. These genotypes were selected with future aim of improving cowpea production for farmers with low income in north-east region. Currently, the cowpea diversity and relatedness among these genetic lines is poorly understood, a situation that needs to be improved because of the presence of a wealth of germplasm resource for this crop in India. Farmers currently classify their genetic lines on the basis of pod or seed characteristics, productivity, cycle duration, or even a person's name.

MATERIALS AND METHODS

Forty-eight cowpea plant materials including varieties

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and cultivars were selected from the germplasm available at the research farm of the Indian Institute of Vegetable Research, Varanasi for the present study (Table 1). Three individual plants per genotype were selected and leaf samples were collected for DNA extraction from 15-day old seedlings. The leaves were frozen in liquid nitrogen and stored at -80°C until used.

Field evaluation of cowpea genotypes was conducted at the research farm of Indian Institute of Vegetable Research, Varanasi, India during 2013 and 2014. Seeds were sown on the raised bed at 30 cm plants to plant and 60 cm apart from row spacing and 14-16 plants were maintained in each row. The experiment was laid out in a complete randomized block design with three replications, each replication was represented in two row plot. Ten plants were randomly taken and tagged for recording observations. The morphological data were recorded on days to 50% flowering, plant height (cm), number of primary branches/plant, peduncle length (cm), number of peduncles/plant, number of pods/plant, pod length (cm), pod weight (g), number of seeds/pod, pod yield/plant (g), pod colour, seed colour, and cowpea golden mosaic virus disease severity. Pods were harvested at marketable maturity. The statistical analysis was based on pooled data differential scales of measurements for different quantitative traits were minimized by standardizing the data for each trait separately prior to cluster analysis following the STAND module of NTSYS-pc10.

Total genomic DNA was extracted from fresh young leaves collected from four individual plants of each genotype, following cetyl-trimethyl ammonium bromide (CTAB) extraction method with required modification (Doyle and Doyle 1990). The quality of the DNA was checked on 1% agarose gel and Nano drop-1000 spectrophotometer.

The SSR analysis was performed with 40 primers supplied by Operon Technologies Inc. California, USA (Table 1) following known protocol with minor modifications (Rai *et al.* 2015). The PCR amplification was carried out in 25µl reaction mixture, containing 15 ng genomic DNA, 2.5 mm $MgCl_2$, 100 mdNTPs, 10X assay buffer, 0.2 ml primer, and 0.6U of Taq polymerase. Amplification was performed in dome shaped capillary tubes in Bio-Metra Thermal Cycler programmed as one cycle of initial denaturation at 94 °C for 4 min, 40 cycles each of denaturation at 94 °C for 1 min annealing at 55-60 °C for 1 min and elongation for 1 min at 72 °C and final elongation at 72 °C for 10 min. The amplified products were separated by electrophoresis in 2.5% (W/V) agarose gels, stained with ethidium bromide and

Table 1 List of primer combinations and their polymorphism information content and resolving power used in this study

Primer codes	Primer sequences	Number of alleles	PIC	% Polymorphism	RP
VM-4	AGTAAATCACCCGCACGATCGAGGGGAAATGGAGAGGAT	5	0.86	85.8	2.66
VM-5	AGCGACGGCAACAACGATTTCCCTGCAACAAAAATACA	1	0.44	44.0	0.5
VM-8	TGGGATGCTGCAAAGACACGAAAACCGATGCCAAATAG	2	0.70	70.0	1.20
VM-9	ACCGCACCCGATTTATTTCATATCAGCAGACAGGCAAGACCA	1	0.45	45.0	0.50
VM-10	TCCCACTCACTAAAATAACCAACCGGATGCTGGCGGCGGAAGG	2	0.70	70.0	1.20
VM-11	CGGGAATTAACGGAGTCACCCCCAGAGGCCGCTATTACAC	4	0.41	41.0	0.87
VM-12	TTGTCAGCGAAATAAGCAGAGCAACAGCAGACGCCCAACT	1	0.45	45.0	0.50
VM-14	AATTCGTGGCATAGTCACAAGAGAATAAAGGAGGGCATAGGGAGGTAT	1	0.45	45.0	0.50
VM-15	CGGCTGCAGCAAACAAGAGAAACCCGTGCAAGAAACCAA	3	0.70	70.0	2.33
VM-16	TCCTCGTCCATCTTCACCTCACAAGCACCGCATTAAAGTCAAG	1	0.63	63.0	0.37
VM-17	GGCCTATAAATTAACCCAGTCTTGTGTCTTTGAGTTTTTGTTCTAC	1	0.45	45.0	0.50
VM-18	AGCCGTGCACGAATGATTGGCCTCTACAACAACACTCT	4	0.47	47.0	2.08
VM-19	TATTCATGCGTGACACTATCGTGGCACCCCCTATC	1	0.47	47.0	0.45
VM-20	GGGGACCAATCGTTTCGTTCATCCAAGATTCGGACACTATTCAA	1	0.45	45.0	0.50
VM-21	TAGCAACTGTCTAAGCCTCACCAACTTAACCATCACTCAC	1	0.71	71.0	0.54
VM-24	TCAACAACACCTAGGAGCCAAATCGTGACCTAGTGCCCACC	3	0.65	65.0	0.5
VM-25	CCACAATCACCGATGTCCAACAATTCCACTGCGGGACATAA	3	0.41	41.0	0.87
VM-26	GGCATCAGACACATATCACTGTGTGGCATTGAGGGTAGC	2	0.16	16.0	0.25
VM-27	GTCCAAAGCAAATGAGTCAATGAATGACAATGAGGGTGC	1	0.18	18.0	0.23
VM-28	GAATGAGAGAAGTTACGGTGGAGCACGATAATATTTGGAG	3	0.45	45.0	0.62
VM-30	CTCTTTCGCGTTCCACACTTGCAATGGGTTGTGGTCTGTG	1	0.34	34.0	0.45
VM-34	AGCTCCCCTAACCTGAATTAACCCAATAATAAGACACAT	1	0.21	21.0	0.54
VM-36	ACTTTCTGTTTTACTCGACAACTCGTCGCTGGGGGTGGCTTATT	1	0.69	69.0	1.13
VM-37	TGTCCGCGTTCTATAAATCAGCCGAGGATGAAGTAACAGATGATC	1	0.75	75.0	1.92
VM-38	AATGGGAAAAGAAAGGGAAGCTCGTGGCATGCAGTGTCAG	2	0.12	12.0	0.25
VM-39	GATGGTTGTAATGGGAGAGTCAAAAGGATGAAATTAGGAGAGCA	1	0.76	76.0	1.79
VM-40	TATTACGAGAGGCTATTTATTGCACTCTAACACCTCAAGTTAGTGATC	1	0.67	67.0	1.61

photographed under ultra- violet light with Alfa InfoTech.

Differences in the DNA banding patterns were qualitatively scored from gel photographs for presence (1) and absence (0) of bands assuming that each band represents a unique genetic locus. Homology of bands among samples was based on the distance of migration in gel. Scoring was done for clear, unambiguous amplicons and their sizes were determined by comparing with 100 bp DNA ladder. Based on the presence or absence of amplicons, a binary 1-0 data matrix was created on Excel sheet and used to calculate Jaccard's similarity coefficient (Jaccard 1908). Cluster analysis was carried out among the genotypes based on Jaccard's similarity coefficients using UPGMA and SAHN-clustering algorithm in NTSYS-pc, version 2.02e software.

Popgen version 1.0 (Marshall *et al.* 1998) calculated polymorphic information content (PIC), observed and expected heterozygosity, allele frequencies, and tested Hardy–Weinberg equilibrium at each locus and for specific groups. Botstein *et al.* (1980) originally defined PIC values as the probability of a given marker being informative in a random mating.

RESULTS AND DISCUSSION

SSR polymorphism

Study of genetic variation in plants is of utmost importance for germplasm maintenance by identifying alleged redundancies and genetic impurity by developing core collection that can be a source of raw material for farmers and breeders to improve productivity through plant breeding. This technique can be a valued contrivance for maintaining various crops including cowpea, as a matter of fact in India, the naming of genotypes by traditional farmers is problematic. Local varieties and genotypes are usually named based on morphological characteristics such as color, size, time to maturity, productivity, introducer or locality name, etc. Therefore, variety/cultivar having similar morphological characters may have different names depending on the localities or ethnic groups (Cieslarova *et al.* 2010). The level of polymorphism detected in our study was low which is in agreement with previous series reported by several cowpea researchers which may be due to the hindrance induced by a single domestication event in this crop (Li *et al.* 2001, Tosti and Negri 2002, Badiane *et al.* 2004, Diouf and Hilu 2005) in addition to its inherent mechanism of self-pollination.

A set of 40 pairs of primers (Table 1) designed to amplify known SSR regions in cowpea were used to analyze genetic diversity of 48 cowpea genotypes including varieties and cultivars. However, only 27 of the SSRs primer amplified in all the 48 samples out of which 11 SSR primers showed clear polymorphic bands. Sixteen SSR primers did not show any polymorphism between varieties and therefore, they were excluded in the analysis. The primers VM-4 amplified the highest (5) and VM-5 lowest (1) number of alleles, respectively across the DNA samples. All the 11 SSR markers showed polymorphism among the cowpea accessions thereby confirming their usefulness for genetic analysis. The genetic diversity of the sample varied from 0.02 to 0.39 (mean = 0.205). The polymorphic information content ranged from 0.12 to 0.86 (mean = 0.49). However, there are various finding that had reported more number of allele in cowpea collected from Senegal in which allele ranged from 1 to 9 (Diouf and Hilu 2005). In contrast, Asare *et al*. (2010) reported 4 to 13 alleles in cowpea collected from Ghana, while Sawadogo *et al.* (2010) reported 5 to 12 alleles in cowpea collected from Burkina Faso using cross species SSRs from Medicago. These findings were in agreement with recent reports on the number of alleles detected using SSR makers in other legumes, such as, 9 to 14 in alfalfa (Mengoni *et al*. 2000), 1 to 9 in yardlong bean (Tantasawat *et al*. 2010) and 3 to 12 in pea (Sarikamis *et al*. 2010). The products amplified by primers VM-5 and VM-4 showed a high similarity with other microsatellites primer used in *Phaseolus vulgaris*, *Lens culinaris*, *Medicago truncatula, M. sativa*, *Pisum sativum* suggesting a synteny between these species and cowpea. These findings should help to quickly identify these genes since the whole genome of some of these legume crops has been sequenced. In fact, most of the SSR primers used in this study were derived from the sequences that are homologous to resistance genes or SSRs in ESTs.

Genetic variation among genotypes

All the 48 genotypes based on their molecular markers, were mainly divided into two clusters with 45 and 3 (KVCP-1, KVCP-2, Ujjain AC) genotypes at the first node (Fig 1). Cluster with 45 genotypes was again divided into two sub-groups at second node with 2 (KPC-11, KVCP-5) and 43 genotypes. Similarly, 43 genotypes were further subdivided into two groups at the third node with 39 and 4 genotypes and 39 genotypes were again divided into two groups at the forth node with 35 and 4 genotypes. On the basis of morphological characters, all the 48 genotypes were also divided in to two clusters with 45 and 3 genotypes (Deharadun AC, Jaipur AC 1, Jaipur AC 2) at the first node (Fig 2). Cluster with 45 genotypes were again subdivided into two sub-group at the second node with 28 and 17 genotypes. Both the cluster of 28 and 17 genotypes were subdivided into two subgroups with 13 and 15 genotypes and 11 and 6 genotypes respectively. Since these clusters are groups of individuals possessing similar characters mathematically gathered into the same cluster, these individuals are supposed to to exibhit higher external hetrogenity. Genotypes included in the same clusters with a high order of divergence will be expected to provide the best breeding materials for achieving the maximum genetic advance for yield per se, provided other factors do not operate to limit the realization of this potential. It is rather encouraging that the divergence revealed in the present genotypes due to these characters will offer a good scope of improving pod yield through rational selection. A crossing program involving parents selected on the basis of genetic divergences of yield component may produce transgress

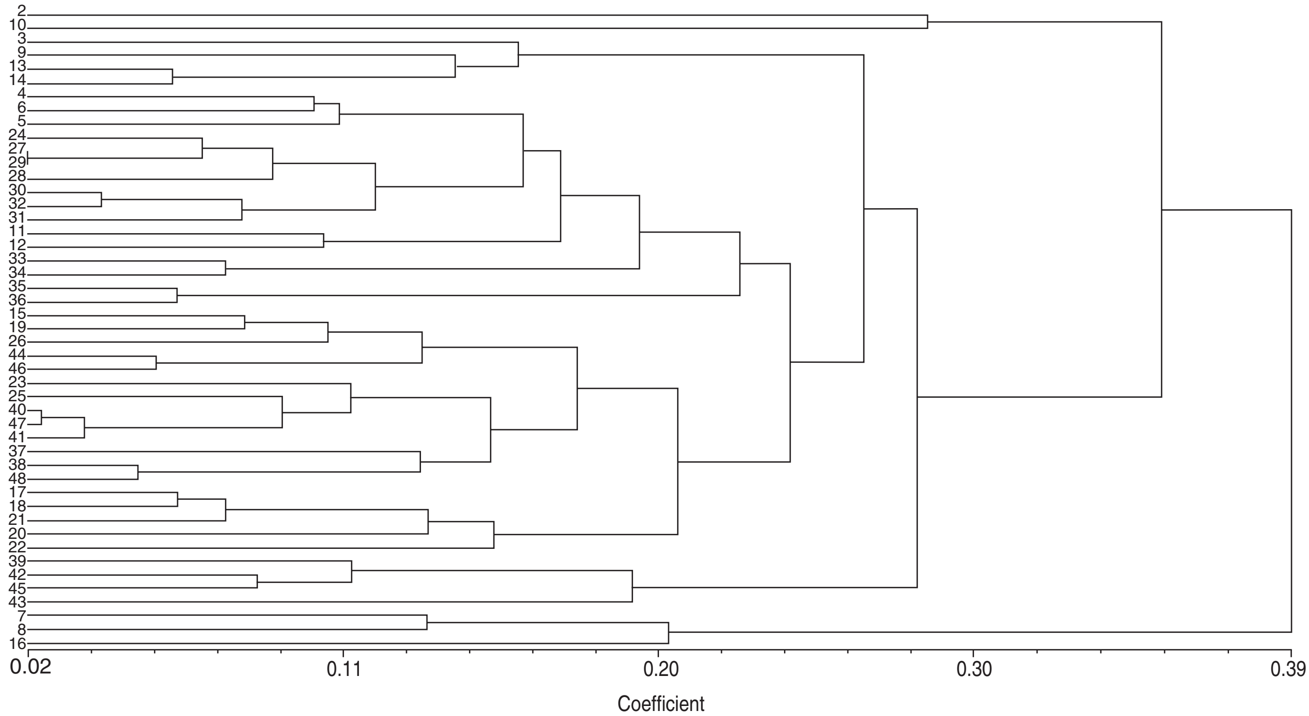

Fig 1 Dendogram showing similarities between 48 cowpea genotypes based on microsatellite markers.

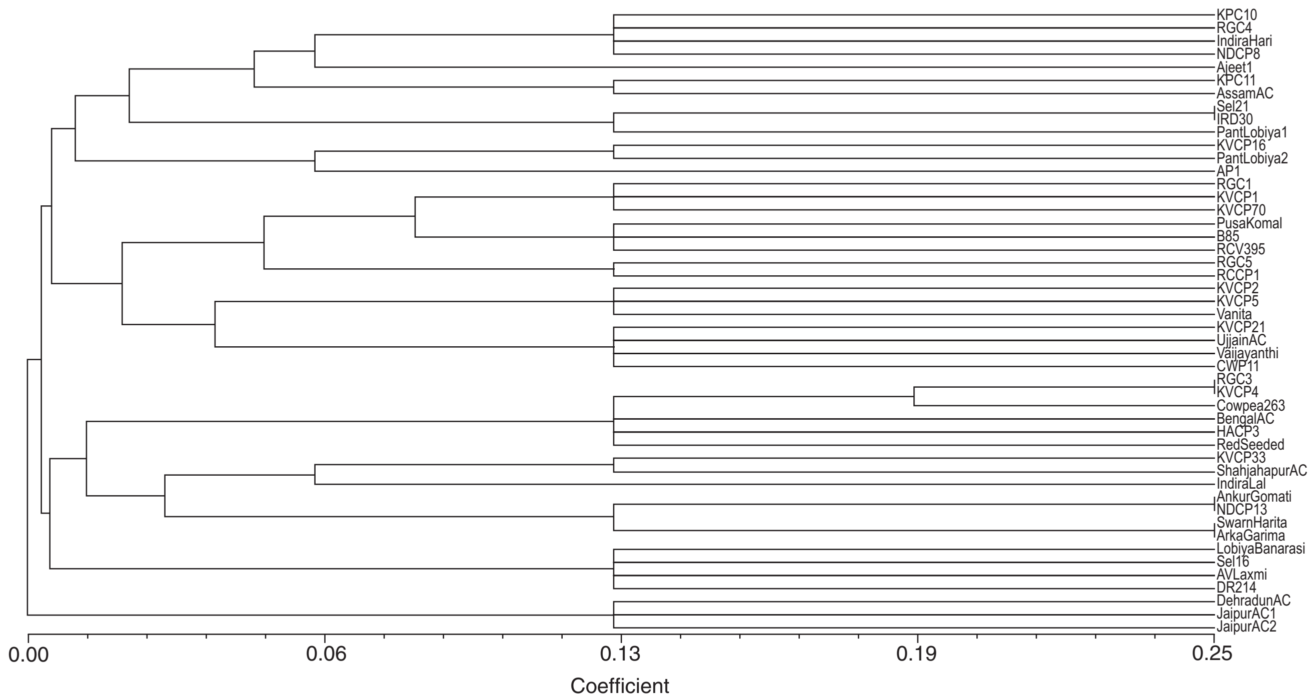

Fig 2 Dendogram showing similarities between 48 cowpea genotypes based on morphological traits.

segregates for yield potential. These results were in close conformity with the finding of Hazra *et al*. (1992). Future investigation need to include a wider number Indian germplasm and perhaps additional informative SSR markers to assess the genetic relationship among genotypes for a rational exploitation in breeding improved varieties.

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Table 2 Morphological characteristics of 48 cowpea genotypes evaluated in the study

Name of the accessions	Days to 50% flowering (DAS)	Plant height (cm)	Primary branches/plant (No)	Peduncle length (cm)	Peduncles/ plant (No)	Pods/ plant (No)	Pod length (cm)	Pod weight (g)	Seeds/ pod (No)	Pod yield/ plant (g)	Podcolour	Seed colour
KPC-10	59.2	186.3	4.2	26.3	14.7	19.9	21.4	7.5	13.7	144.8	Light green	Grey and cream
KPC-11	50.3	190.2	3.6	29.4	16.6	23.7	34.4	12.5	15.1	290.3	Light green	Black
RGC-1	62.3	177.2	4.1	38.4	15.7	22.5	16.9	5.5	12.8	126.2	Light green	Red and cream
RGC-3	52	149.5	3.5	28.4	16.8	21.5	23.0	7.5	13.4	164.5	Light green	Grey and cream
RGC-4	49.5	190.2	4	29	14.7	18.4	38.4	14.5	12.9	261.4	Light green	Red
RGC-5	51	76	5.5	23.1	22.2	32.7	15.6	4.5	9.4	142.7	Green	Red
KVCP-1	50	268	4.3	32.3	14.2	17.7	24.5	5.5	12.9	99.3	Red	Red and cream
KVCP-2	49.8	255.8	4.2	30.6	15.1	18.6	38.6	14.0	11.8	252.6	Light green	Red
KVCP-4	47.5	183.4	3.5	28.4	10.4	14.4	26.7	6.5	17.7	91.7	Green	Red
KVCP-5	50.2	179	4.1	30.2	15.1	24.8	39.2	15.0	13.7	364.5	Dark green	Red
KVCP-16	49.5	165	3.7	32.3	15.2	19	22.4	5.1	15.3	93.9	Light green	Red
KVCP-21	47.2	181.6	4.1	30.4	17	19.9	30.2	12.0	13.5	231.6	Light green	Red
KVCP-33	54.3	216.8	3.8	40	14.4	17.5	24.6	10.0	13.1	171.5	Green	Black
KVCP-70	45.5	198.6	4.1	35.5	14.2	22.8	40.4	13.5	14.2	313.8	Green	Red
Assam AC	45.2	214.8	4.2	29.4	17	20.8	18.5	6.4	12.1	130.4	Light green	Brown
Ujjain AC	55.3	248.5	4.1	36.8	15.5	23.2	37.7	9.8	16.3	231.9	Green	Red
Bengal AC	51.7	218.9	3.5	32.3	16.7	25.1	40.3	12.7	15.6	309.2	Green	Red
Shahjahapur AC	46	102.3	4.4	30.8	18	17.5	20.7	7.5	10.4	133.8	Light green	Brown
Dehradun AC	50.2	144.6	4.6	40.7	11.8	18	46.0	12.1	19.0	213.4	Dark green	Red
Jaipur AC-1	40.5	59.6	4.6	43	25	49.6	12.2	2.5	10.0	121.3	Dark green	Cream
Jaipur AC-2	48.5	146.8	4.6	26.6	15.5	19.3	27.0	10.1	14.6	189.1	Green	Brown
Indira Lal	51.2	285.9	4	30.8	14.6	17.3	24.2	8.5	14.7	144.1	Red	Red and brown
Indira Hari	53.3	217.5	4.2	32.3	14.9	18.4	28.7	9.7	13.7	173.2	Light green	Cream
Ankur Gomati	55.4	129.8	4.3	32.4	14.4	22.2	23.5	6.0	14.2	135.9	Light green	White and grey
Sel. 2-1	44	64.3	4	22.4	21	23	21.1	8.1	9.9	182.6	Light green	Black
IRD-30	44	82.8	3.2	31.5	21	28	14.0	3.6	9.8	97.8	Green	Cream
Lobiya Banarasi	43.3	148.9	3.4	28.2	17.8	20.2	14.0	4.8	11.4	98.8	Green	Brown
HACP-3	61.6	223.8	3.5	30.3	11.9	13.2	27.6	8.0	15.5	103.5	Cream	Red
Swarn Harita	51.5	219.1	4.3	30.1	13.3	17.4	26.8	8.0	13.4	135.0	Green	Red
Pant Lobiya-1	44	56.4	3.6	24.2	12.2	15.8	13.2	3.5	6.8	56.4	Light green	Cream
Pant Lobiya-2	49.5	40.8	4.8	32.8	18.8	30.5	13.0	3.4	7.7	101.6	Light green	Red
Vanita	45.8	57.9	4.4	32.4	15.1	24	21.3	6.1	8.8	142.2	Light green	Black
NDCP-8	43.2	169.4	3.2	18.2	14.7	14.6	27.8	9.7	11.2	147.5	Dark green	Red

Contd.

Table 2 (*Concluded*)

Name of the accessions	Days to 50% flowering (DAS)	Plant height (cm)	Primary branches/plant (No)	Peduncle length (cm)	Peduncles/ plant (No)	Pods/ plant (No)	Pod length (cm)	Pod weight (g)	Seeds/ pod (No)	Pod yield/ plant (g)	Podcolour	Seed colour
NDCP-13	50.3	130.2	4.3	31.4	15.4	20.9	22.4	6.0	12.3	127.9	Light green	Black
Pusa Komal	46	47.6	5.6	34.8	30	28.3	17.2	5.5	11.2	204.3	Light green	Cream
Cowpea-263	43	58.3	3.5	31.4	25.2	33.6	22.2	7.5	9.6	257.4	Green	Cream
Arka Garima	51.5	242.8	4.3	17.6	14.8	17.5	19.3	5.8	8.4	99.5	Light green	Brown
Sel-16	50	43.2	3.4	24.8	16.5	23.4	21.3	7.9	9.6	179.3	Light green	Brown
AP-1	49	79.4	3.7	37.3	14.5	21.3	16.2	5.0	11.8	104.4	Light green	Cream
A. V. Laxmi	61.6	264.3	3.4	25.4	33.4	24.3	15.8	6.9	9.3	171.0	Light green	Greenish cream
SB-5	48	128.5	4.7	32.7	12.2	18.1	21.7	5.5	14.8	96.5	Light green	Red
DR-214	56	139.5	3.4	31.4	15.9	19.2	15.4	4.6	11.4	86.5	Light green	Cream
Vaijayanthi	62.2	263.4	4.1	32.6	14	19.5	37.5	7.0	10.7	139.2	Red	Red
Red Seeded	48.5	155.8	3.5	19.7	14.6	18.2	18.3	9.5	10.7	167.7	Green	Red
CWP-11	46.3	73.8	4.1	28.6	17.2	18.4	20.4	7.7	13.3	138.8	Light green	Cream
RCV-395	50.4	53.3	3.9	26.2	18.3	20.2	15.7	5.5	12.1	113.2	Light green	Cream
RCCP-1	51	94.4	5.2	34.9	19.7	23.3	14.7	5.5	15.3	124.3	Green	Black
Ajeet-1	45.7	64.3	4.2	41.6	22.2	35	16.8	4.0	12.7	142.8	Green	Cream

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