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# Novel chloroplast microsatellite markers in pigeonpea (*Cajanus cajan* L. Millsp.) and their transferability to wild *Cajanus* species

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**Abstract**

Chloroplast microsatellites are potential genetic markers which provide insight into plant systematics studies. A set of 39 new chloroplast microsatellite markers were developed by analyzing the pigeonpea chloroplast genome sequence. Primer pairs were designed for 39 random sequences containing mononucleotides, dinucleotides, tetranucleotides and compound repeat motifs. The newly developed chloroplast SSRs were checked for their transferability in six wild *Cajanus* species (one accession each). 17 of the 39 cpSSRs displayed polymorphism among the wild *Cajanus* species with an average polymorphism information content (PIC) value of 0.40. In total, 41 alleles were produced at the polymorphic loci, each marker generating on an average 2.41 alleles per locus. This point towards the effectiveness of the primer pairs in detecting genetic relatedness amid species within the genus *Cajanus*. Genetic relationship based on neighbor-joining method revealed two major groups (Group I and group II) of which Group II consisted of two main clusters including the cultivated *Cajanus cajan* and other wild *Cajanus* species except for one wild species *C. platycarpus* that was most diverse from rest of the species.

**Keywords:** Cluster analysis; genetic diversity; phylogenetic study; polymorphism; SSRs; wild relatives.
**Abbreviations:** AFLP_amplification fragment length polymorphism; CMS_cytoplasmic male sterility; cpSSRs_chloroplast simple sequence repeats; CTAB_cetyl trimethyl ammonium bromide; EtBr_ethidium bromide; ISSR_inter simple sequence repeats; PIC_polymorphism information content; RAPD_randomly amplified polymorphic DNA; RFLP_restriction fragment length polymorphism; SSRs_simple sequence repeats.

## Introduction

Pigeonpea {*Cajanus cajan* (L.) Millsp.} is a vital legume crop which belongs to the family *Fabaceae* with an estimated 858 Mbp genome size (Greilhuber and Obermayer, 1998) and is often cross-pollinated. Pigeonpea is the second major grain legume crop holding an important position in the rain-fed agriculture (Saxena et al., 2010). India is considered as the center of origin of pigeonpea (Van der Maesen, 1980) which then successfully traveled to East Africa and also to the American continent. The three major pigeonpea producing regions are the Indian sub-continent, central-America and eastern Africa. It is well known as a protein-rich crop with 21% protein content in the seeds along with the presence of various vital amino acids (Dutta et al., 2013). Pigeonpea is globally cultivated on 5.32 Mha land area with an overall annual production of 4.32 Mt (FAO 2013). The states of Maharashtra, Madhya Pradesh, Gujarat, Uttar Pradesh, Karnataka and Andhra Pradesh account for over 70% of the total pigeonpea area in India (Saxena and Nadarajan, 2010). In the past five decades, a steady growth in production of pigeonpea has been recorded; however average yield remains static at around 700kg/ha (Nadarajan et al., 2008).

Pigeonpea is greatly influenced by different biotic and abiotic factors which restrain its yield capacity. The resilience to all these challenges is absent in the cultivated genotypes but on the other hand, few of the wild relatives offer strong resistance to these constraints (Mallikarjuna et al., 2011). The wild species of pigeonpea possess various desirable attributes such as high protein content, dwarf growth habit and cytoplasmic male sterility (CMS) (Saxena et al., 2002; Saxena and Sharma, 1995; Mallikarjuna and Saxena, 2005; Saxena et al., 2005). Extensive morphological variations exist within cultivated species of pigeonpea but are not available as they display low levels of polymorphism. In contrast, the wild relatives of pigeonpea provide a vital source of genetic variability with respect to resistance to diseases, pest and drought (Panguluri et al., 2006).

Estimation of genetic diversity and its utilization in the breeding programme is of key importance for crop improvement. In pigeonpea, morphological and biochemical (isozymes) markers have been previously employed for the estimation of genetic diversity of the cultivars and the wild species but these markers are influenced by environmental conditions and are not considered as a reliable source for genetic diversity estimation (Ratnaparkhe et al., 1995). In contrast, the desirable attributes of DNA molecular markers such as highly polymorphic, co-dominant inheritance,

frequent distribution in the genome, high reproducibility, easy availability and neutral to the environmental conditions have made them a versatile tool to measure genetic relationship in crop plants (Joshi et al., 1999). Several DNA markers including RFLP (Nadimpalli et al., 1993), RAPD (Ratnaparkhe et al., 1995), AFLP (Panguluri et al., 2006) and ISSR (Yadav et al., 2014) were successfully employed for estimation of intra-specific and inter-specific genetic diversity and phylogenetic studies in pigeonpea. In past years, SSRs have gained significant importance and are widely accepted class of molecular markers for genetic analysis of crop plants (Oliveira et al., 2006).

Simple sequence repeats (SSRs) or microsatellites are short DNA stretches of single specific loci containing one to six bases which are tandemly repeated (Schlotterer, 2000). Eukaryotic genomes are densely interspersed with simple sequence repeats which exhibit a high degree of polymorphism as a result of length variation due to the occurrence of a different number of repeat units (Morgante and Olivieri, 1993). Microsatellites are preferred DNA markers as these are abundant in the genome, multiallelic nature, highly variable, co-dominant inheritance, reproducibility and amenability to automation and high throughput genotyping (Powell et al., 1996). In similar fashion to nuclear SSRs, chloroplast microsatellites (cpSSRs) also exhibit polymorphism due to length variability as consequences of alterations in the number of repeat motifs (Powell et al., 1995).

Chloroplast simple sequence repeats (cpSSRs) are generally located in the non-coding regions inclusive of introns and intergenic spacers of the cp genome. Chloroplast SSRs show remarkable intra-specific variation and thus are contemplated as efficient markers in the evolutionary and systematic investigation in plants (Provan et al., 2001). Till date, 191 SSRs have been identified in pigeonpea through enrichment of genomic libraries (Burns et al., 2001; Odeny et al., 2007, 2009). With advancement in the sequencing technologies, 3072 SSRs were reported using BAC end sequencing (Bohra et al., 2011). Deep transcriptome studies further identified 3583 and 3771 genic SSRs in pigeonpea (Raju et al., 2010; Dutta et al., 2011). A total of 189,895 genomic SSRs have been added to the previously available SSRs with the release of the pigeonpea draft genome sequence (Singh et al., 2012). From the above studies, a complete set of genic and genomic SSR markers has been developed as a significant genomic tool for diversity assay in pigeonpea. However, till date development of chloroplast SSR markers (cpSSRs) has not been reported in pigeonpea. In the current study, the cpSSRs so developed were used for discerning genetic relationship among *Cajanus cajan* and six wild *Cajanus* species.

## Results

### *Microsatellite mining, development of cpSSR markers and their genetic assessment*

Pigeonpea chloroplast genome sequence (accession number: KU729879) was used for SSR mining (Kaila et al., 2016). A total of 292 chloroplast SSRs (cpSSRs) were mined in pigeonpea using MISA perl script. Primer sets flanking 39 SSR loci were designed and designated as *Cajanus cajan* chloroplast (Cccp) SSR markers (Table 1). One accession each of wild *Cajanus* species including *C. cajan* was utilized for testing cross-species amplification of the newly developed chloroplast SSRs. All the cpSSR markers demonstrated scorable results with 100% transferability efficiency. 17 of the 39 Cccp SSR markers were polymorphic (43.5%) and remaining was found to be monomorphic. A PCR amplification profile produced by one of the polymorphic markers is given in Fig. 1. Overall, 41 alleles were generated by the polymorphic loci with an average of 2.41 alleles for each locus. The number of alleles at these loci varied from 2 (Cccp_03, Cccp_04, Cccp_05, Cccp_10, Cccp_11, Cccp_14, Cccp_23, Cccp_28, Cccp_32, Cccp_35, Cccp_37, Cccp_39) to 5 (Cccp_08). The discriminatory competence of each SSR marker was evaluated by PIC values that varied from 0.21 to 0.71 (average = 0.40). Likewise, the major allelic frequency for these polymorphic loci varied from 0.43 (Cccp_08 and Cccp_13) to 0.87 (Cccp_11, Cccp_23, Cccp_28, Cccp_32 and Cccp_39) with average of 0.68 (Table 2). Majority of the polymorphic loci include mononucleotides (47%), dinucleotides (29.4%), one tetranucleotide and three compound repeats.

### *Phylogenetic study of Cajanus cajan and wild Cajanus species*

The polymorphic cpSSR markers were efficient in differentiating the individual genotypes in the present study. The allelic data generated by them was utilized to compute Nei's genetic distance values and to construct a dendrogram (Fig. 2). The neighbor-joining cluster analysis reported two groups; Group I and Group II with 100% bootstrap values suggesting complete support to the grouping pattern. Group I, includes only one genotype that was distinctly different from other genotypes forming Cluster I. Group II was divided into two clusters of which Cluster II and Cluster III contained 3 genotypes each. Cluster I represent only single wild species *Cajanus platycarpus* which was an outlier. Cluster II contained *Cajanus lineatus, Cajanus acutifolius* and *Cajanus sericea* (bootstrap values varied from 7% to 20%) on the other hand Cluster III contained *Cajanus cajan* and wild species *Cajanus cajanifolius* and *Cajanus scarabaeoides* (bootstrap values 50%).

## Discussion

### *Mining of cpSSR markers and their utilization in genetic assessment*

Chloroplast microsatellites (cpSSRs) are extremely useful due to the conserved gene order, uniparental and non-recombinant inheritance of the chloroplast genome (cpDNA) making these proficient for phylogenetic and evolutionary studies in plants (Olmstead and Palmer, 1994). Previously, a large set of genic and genomic SSRs employed for genetic variability studies in pigeonpea were reported (Burns et al., 2001; Odeny et al., 2007, 2009; Raju et al., 2010; Bohra et al., 2011; Dutta et al., 2011; Singh et al., 2012). The number of cpSSR mined in the present study was higher than the ones identified in *Sesamum indicum*, *Vigna radiata* and *Camellia* species (Yi and Kim, 2012; Lin et al., 2015; Huang et

**Table 1.** List of cpSSRs primer pairs used for genetic relationship studies among pigeonpea and its wild relatives.

| S.No | Primer Id | Repeat motif | Left Primer Sequence 5'-3' | Right Primer Sequence 5'-3' | Allele size/range (bp) | Tm°C |
|---|---|---|---|---|---|---|
| 1 | Cccp_01 | (A)11 | CAGTTTATGCATGGCGAAAG | GCAACAACTGGTACCATAGA | 217 | 52 |
| 2 | Cccp_02 | (A)13 | ACGTGATGATATAGCCCAAC | ACCTGTTCTTTCCATGACTC | 140-200 | 52 |
| 3 | Cccp_03 | (T)10 | GGGCTCTTTGACTGTAGAAA | CCATCTCTCCCCAATTGAAA | 180-231 | 55 |
| 4 | Cccp_04 | (A)10 | GATCAAAAGTTTATCCGCGG | CCTTTTCTTGCCCGTATTTC | 245-255 | 55 |
| 5 | Cccp_05 | (A)22 | ATGGTATGTTGCTGCCTTTT | CCTTTTCTTGCCCGTATTTC | 186-195 | 52 |
| 6 | Cccp_06 | (A)14 | ACGCGTAAATAGATTGACCT | TGGAATTGTCATAGTTTTCGA | 242 | 55 |
| 7 | Cccp_07 | (A)20 | AGCATAGAAGAAACCCATGT | GAAACCATTCTTTGAATTCTTCG | 224-330 | 55 |
| 8 | Cccp_08 | (T)13 | CTACTTGGGTATTGAGCGTT | TAGATCCATCCTCCGAAAGA | 150-260 | 55 |
| 9 | Cccp_09 | (T)10 | CCGTATCATCTTGACTTGGT | TTGTCAACTACTCCTATCGG | 217 | 53 |
| 10 | Cccp_10 | (T)12 | TTTTCACCTCCAATCCAACA | AGAGTGCTTCAAAATTCAAACT | 215-232 | 54 |
| 11 | Cccp_11 | (T)16 | GTTCCCCAACTAGAATGTGT | GGTTGTTGTTATCTGCTCCA | 159-170 | 55 |
| 12 | Cccp_12 | (AT)5 | CCTAGATACCCGCTTTTTCT | GGCTTTCTATCTTTGGGATT | 205 | 55 |
| 13 | Cccp_13 | (AT)9 | ATCATGTCATGTCGATTGTG | TTGAAATCTGTTGGGATAGG | 195-230 | 56 |
| 14 | Cccp_14 | (TA)5 | CATTTCATAGGGAACCTCAA | TTTTCAACTTTCCATTTTCC | 150-160 | 54 |
| 15 | Cccp_15 | (AT)5 | TTTTCAACTTTCCATTTTCC | CATTTCATAGGGAACCTCAA | 159 | 54 |
| 16 | Cccp_16 | (CT)5 | CTGGATATCTGTTCCCCATA | TGAAAAATGAAAAGGTCGTC | 173 | 52 |
| 17 | Cccp_17 | (AT)5 | CATATAGATCCCTGCGTTGT | TGGTTCGATTTGAATTATCC | 215 | 55 |
| 18 | Cccp_18 | (ATAG)3 | TTCCGAATGGAATAAAAGAG | AAACCCAAATGAACAAAATG | 160-240 | 53 |
| 19 | Cccp_19 | (A)12 | CCACATCAAGCACTAACCTC | GCATGTCGTATCAATGAAGA | 242 | 54 |
| 20 | Cccp_20 | (A)11 | TGTCATTACGTGCGACTATC | AGAAAAAGCGGGTATCTAGG | 246 | 55 |
| 21 | Cccp_21 | (A)10 | CAGGTTCGAATTCCATAGAG | GCAAGTTGATCGGTTAATTC | 232 | 55 |
| 22 | Cccp_22 | (T)10 | GGTAGAGCACCTCGTTTACA | CATTGACGAGAAATGGGTAT | 224 | 55 |
| 23 | Cccp_23 | (A)10 | TTTAGGAGATTACCCATTCG | AGCAAAGGGTTTTCACTTTC | 180-221 | 55 |
| 24 | Cccp_24 | (T)11 | GATACGTAAGCAAGGCATTT | ATTTTTCTTCGGAGAGTTCC | 224 | 53 |
| 25 | Cccp_25 | (G)10 | AGGGCAATCACTCATTCTTA | TCCAAAGAGCAACTCTTCTC | 192 | 54 |
| 26 | Cccp_26 | (A)14 | AACTACTGGAGGGAAAAAGG | AACTGTTTTACGCCTTTGAG | 236 | 51 |
| 27 | Cccp_27 | (C)10 | TCCAAAGAGCAACTCTTCTC | AGGGCAATCACTCATTCTTA | 192 | 55 |
| 28 | Cccp_28 | (TA)5 | GACCTTCCAAAATCCTTCTT | GGTTTTTCGTCAATTCCAG | 226-280 | 57 |
| 29 | Cccp_29 | (AT)5 | GCATCTTGAAAGTGAATCGT | GGCTTCTATTGAATCGAGAA | 239 | 52 |
| 30 | Cccp_30 | (AG)5 | AGCTTAACACCTCTCATTCCT | CTGGATATCTGTTCCCCATA | 226 | 53 |
| 31 | Cccp_31 | (TA)6 | ATACACCCTGGTACACGTTC | AAGTAATTCGGGGTTAGGAT | 183 | 54 |
| 32 | Cccp_32 | (AT)6 | CTGCCGTTTTTATAGTTTCC | TATCAAGAGACCTGCCAAAG | 236-270 | 52 |
| 33 | Cccp_33 | (A)8N(AT)5N(AAAT)3 | TTGAAAACCGGTATAGTTCG | TGAAAAAGGAATTGATCGAG | 318 | 56 |
| 34 | Cccp_34 | (AT)5N(TA)6 | AGGCCTTATCCACACAAGTA | GCGGGTATCTAGGCATATCT | 351 | 56 |
| 35 | Cccp_35 | (TTA)5N(ATT)5N(A)8 | TAAACCAATTTGCGTCCA | AGAATTATAGAATGAATCCAAA | 310-325 | 55 |
| 36 | Cccp_36 | (T)16N(A)8 | TGATGAATCTTCCATTTTCA | TCAATGACCGAGAATTGTAA | 321 | 53 |
| 37 | Cccp_37 | (T)13N(A)14(AATA)3N(A)10N(AT)6N(A)8 | TGAAAAATTAATATGGATTACTG | AGGGGGTTTTCTATATTTTCTT | 230-250 | 55 |
| 38 | Cccp_38 | (T)10N(A)8 | AATGGTTGTTTCTCCCAAG | TGGTGTTTCTAACCATCCAT | 183 | 54 |
| 39 | Cccp_39 | (T)9N(TTTA)3 | CTCAACCTATTTGAATTTTGG | TTTTATCGGACGGTTCTAAA | 130-140 | 52 |

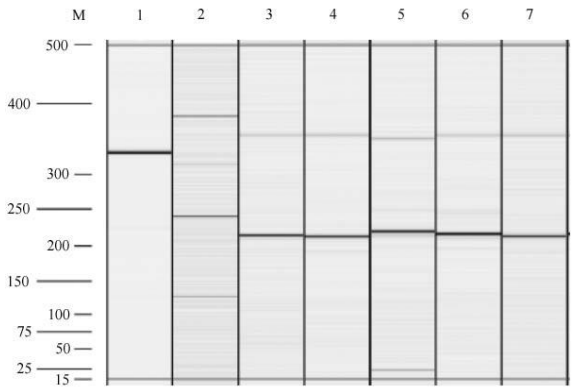


**Fig 1.** PCR amplification profile generated by Cccp_08 SSR marker among six wild *Cajanus* species and *Cajanus cajan*. Lane M- 25-500 bp QX DNA size marker; Lane 1- *C. acutifolius*; Lane 2- *C.cajanifolius*; Lane 3- *C. lineatus*; Lane 4- *C.platycarpus*; Lane 5- *C.scarabaeoides*; Lane 6- *C.sericea*; Lane 7- *C.cajan*

**Table 2.** Polymorphism among the six wild and one cultivated *Cajanus* species.

| S.No | Marker Name | Number of alleles ($N_a$) | Major allele frequency | PIC value |
|---|---|---|---|---|
| 1 | Cccp_02 | 3 | 0.75 | 0.4 |
| 2 | Cccp_03 | 2 | 0.75 | 0.37 |
| 3 | Cccp_04 | 2 | 0.5 | 0.5 |
| 4 | Cccp_05 | 2 | 0.5 | 0.5 |
| 5 | Cccp_07 | 3 | 0.62 | 0.53 |
| 6 | Cccp_08 | 5 | 0.43 | 0.71 |
| 7 | Cccp_10 | 2 | 0.75 | 0.37 |
| 8 | Cccp_11 | 2 | 0.87 | 0.21 |
| 9 | Cccp_13 | 3 | 0.43 | 0.63 |
| 10 | Cccp_14 | 2 | 0.62 | 0.46 |
| 11 | Cccp_18 | 3 | 0.56 | 0.58 |
| 12 | Cccp_23 | 2 | 0.87 | 0.21 |
| 13 | Cccp_28 | 2 | 0.87 | 0.21 |
| 14 | Cccp_32 | 2 | 0.87 | 0.21 |
| 15 | Cccp_35 | 2 | 0.75 | 0.37 |
| 16 | Cccp_37 | 2 | 0.62 | 0.46 |
| 17 | Cccp_39 | 2 | 0.87 | 0.21 |
| | Mean | 2.41 | 0.68 | 0.4 |

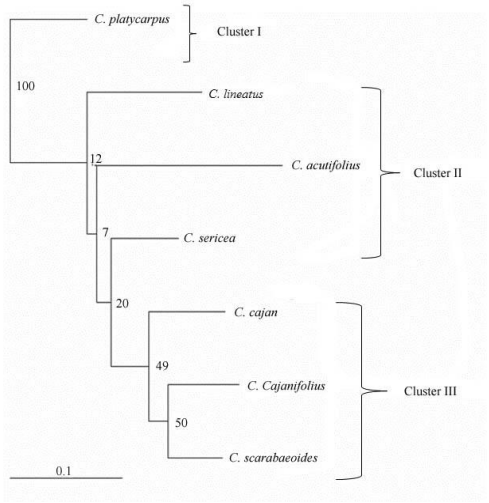


**Fig 2.** Neighbor-joining tree of six wild *Cajanus* species and *Cajanus cajan* obtained by 39 chloroplast SSR markers.

**Table 3.** List of wild *Cajanus* species used for genetic relationship study.

| S. No | CMS System | Species | Accession ID | Source |
|---|---|---|---|---|
| 1. | A1 | *Cajanus sericeus* | ICPW160 | ICRISAT, Patancheru, India |
| 2. | A2 | *Cajanus scarabaeoides* | ICPW87 | ICRISAT, Patancheru, India |
| 3. | A4 | *Cajanus cajanifolius* | ICPW28 | ICRISAT, Patancheru, India |
| 4. | A5 | *Cajanus acutifolius* | ICPW1 | ICRISAT, Patancheru, India |
| 5. | A6 | *Cajanus lineatus* | ICPW44 | ICRISAT, Patancheru, India |
| 6. | A7 | *Cajanus platycarpus* | ICPW68 | ICRISAT, Patancheru, India |

al., 2014). However, the number of SSRs detected in our study was lower in contrast with cpSSRs reported in *Glycine* species. This could be due to the fact that eight *Glycine* species were analyzed for SSR mining. The findings were similar to those reported in *Glycine max* (Ozyigit et al., 2015).

In this study, all the newly developed cpSSRs amplified efficiently and at least one allele was detected in all the accessions indicating their transferability among the wild *Cajanus* species. The amplification efficiency (100%) was higher than those reported by Dutta et al. (2011) and Singh et al. (2012). 43.5% polymorphism was detected by these markers which were higher to 12.9% for genic SSRs, 28.40% for genomic SSRs developed via BAC end sequencing and 40.8% for genomic SSRs previously used for genetic diversity assessment in *Cajanus cajan* (Dutta et al., 2011; Bohra et al., 2011; Singh et al., 2012). This point towards the high and efficient utility of these cpSSR markers in diversity assessment in this crop. The allelic difference observed at the cpSSR loci varies from 2 to 5 alleles with an average of 2.41 alleles per locus. The findings were comparable to previous diversity analysis studies utilizing SSR markers in pigeonpea (Odeny et al., 2009; Bohra et al., 2011; Mudaraddi et al., 2013; Petchiammal et al., 2015). The mean PIC value of 0.40 was demonstrated by these polymorphic markers which were lower to previously reported genic and genomic SSRs in pigeonpea (Dutta et al., 2011; Mudaraddi et al., 2013). The possible reasons could be the utilization of only highly polymorphic SSRs for genetic relationship studies in the previous reports. The PIC value (0.40) in this study was in accordance with the one documented by Odeny et al. (2007, 2009) and higher to ones reported by Petchiammal et al. (2015). These newly developed cpSSRs are therefore competent in genetic diversity, evolutionary and phylogenetic studies in pigeonpea.

***Phylogenetic analysis***

The genetic relatedness among *Cajanus cajan* and six wild *Cajanus* relatives was established using neighbor-joining clustering which revealed two distinct groups: Group I and Group II (Fig. 2). Group I, consisted Cluster I which included only single wild species *Cajanus platycarpus* which was an outlier. *Cajanus platycarpus* was grouped in the tertiary gene pool reason being its low crossability with *Cajanus cajan* (Dutta et al., 2011). This was evident in our study, as *Cajanus platycarpus* was completely distinct from the *Cajanus cajan* and other wild species. Three wild species, namely *C. lineatus, C.acutifolius* and *Cajanus sericea* grouped together in Cluster II. These wild relatives belong to the secondary gene pool and were grouped together in previous reports (Odeny et al., 2007; Dutta et al., 2011). In the study, *C. cajanifolius* showed close relatedness to *C. cajan* and this was supported by the previous study using genomic SSRs (Odeny et al., 2007). *C. cajanifolius* and *C. scarabaeoides* corresponding to the secondary gene pool grouped together in single cluster III, inferring that these two might have common ancestry (Khera et al., 2015). In this study, *C. platycarpus* showed close relatedness to *C. lineatus* and *C. acutifolius* which belong to the secondary gene pool. The finding was similar to the one reported by Mudaraddi et al. (2013) where despite being in the tertiary gene pool genetic variation of *C. platycarpus* was similar with *C. scarabaeoides* and *C. acutifolius* and was grouped together.

These novel cpSSR markers could be used as valuable tools to study diversity analysis and can offer new insights into the origin and phylogenetic relationship within the *Cajanus* genus and other legumes.

**Materials and Methods**

***Plant materials and DNA isolation***

One accession each of *Cajanus cajan* (AKPR375) and six wild *Cajanus* species was used in the present study (Table 3). Seeds of wild species were procured from the International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, India and the plants were maintained in the research farm at Indian Agricultural Research Institute, New Delhi, India. Leaf samples were collected and frozen in liquid nitrogen and stored at -80°C for further use. Genomic DNA was isolated following a modified CTAB method (Saghai-Mahroof et al., 1984). The DNA was analyzed on 0.8% (w/v) agarose gel and visualized by EtBr staining. The DNA concentration was measured by Nanodrop spectrophotometer (Thermo scientific).

***cpSSR mining and primer development***

High-quality chloroplast DNA sequence of *Cajanus cajan* (accession number: KU729879) from GenBank (http://www.ncbi.nlm.nih.gov) (Kaila et al., 2016) was employed for detecting chloroplast microsatellites (cpSSRs) using MISA perl script (MIcroSAtellite, http://pgrc.ipk-gatersleben.de/misa/) (Thiel et al., 2003). We identified cpSSRs as mononucleotide repeats ≥ 8 bases, dinucleotides (five repeats), trinucleotides (four repeats), tetranucleotides (three repeats), pentanucleotide (three repeats) and hexanucleotides (three repeats) respectively. Randomly 39 identified sequences containing microsatellite motifs: mononucleotides (20), dinucleotides (11), tetranucleotides (1) and compound SSRs (7) were used for primer designing using Primer3 plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Untergrasser et al., 2012). Following parameters were used for primer designing: annealing temperature of the primer between 50–60 °C,

primer length (20-22 bases), G+C content ~ 40-50% and the amplicon size varying between 150-350 bp. (Table 1).

***cpSSR amplification and validation***

To test the efficiency of the newly developed cpSSRs, these were employed in genetic relationship studies among one accession each of *Cajanus cajan* and wild *Cajanus* relatives following six divergent CMS system: A1 (*Cajanus sericeus*), A2 (*Cajanus scarabaeoides),* A4 (*Cajanus cajanifolius),* A5 (*Cajanus acutifolius)*, A6 (*Cajanus lineatus)* and A7 (*Cajanus platycarpus)*. Total 20 µl reaction mixture comprised of genomic DNA (50 ng), 1× *Taq* buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.15 µM of each primer, and 0.5 U *Taq* DNA polymerase (NEB). PCR amplification was performed in a thermocycler (Biorad) with an initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 15 s, annealing for 20 s (temperature for each primer mentioned in Table 1) and extension at 72 °C for 20 s followed by final extension at 72 °C for 5 min. The PCR amplified products were analyzed by capillary electrophoresis using QIAxcel electrophoresis system (QIAGEN). 1µl of amplified PCR product was separated on QX DNA High-resolution cartridge along with QX DNA Size marker 25-500 bp v2.0 for sizing of the amplicons and QX DNA Alignment marker 15 bp/1 kb were run simultaneously. Amplicon sizing was performed with the help of QIAxcel ScreenGel software (QIAGEN).

***Phylogenetic study***

To examine the phylogenetic correlation amongst the genotypes included in the study**,** allelic data were scored as present (1) or absent (0). The Nei's genetic distance values were calculated using Free Tree software version 0.9.1.50 (Pavlieek et al., 1999) and the matrix obtained was used for the construction of the neighbor-joining tree. The bootstrap analyses (based on 1000 re-samplings) of the data were performed to evaluate the authenticity of the clustering pattern. Other parameters including major allele frequency and allele numbers were estimated using POPGENE version 1.3 (Yeh and Boyle, 1999). The polymorphic information content (PIC) was derived by using the formula, $PIC=1-\sum pi\ 2$ (Powell et al., 1996), where pi refers to the frequency of the ith allele.

**Conclusion**

A novel set of chloroplast microsatellite markers (cpSSRs) were reported for the first time in pigeonpea. These markers were successfully transferable to six wild *Cajanus* species with the amplification efficiency of 100%. These cpSSRs markers revealed 43.5% polymorphism among pigeonpea and its wild relatives depicting their potential in genetic diversity and phylogenetic studies. These newly developed markers will be beneficial in providing information about evolutionary and phylogenetic relationship within the *Cajanus* genus and other legumes.

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