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Molecular diversity among wild relatives of *Cajanus cajan* (L.) Millsp.

Bharathi Mudaraddi*, K. B. Saxena, R. K. Saxena and R. K. Varshney

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, 502 324 Andhra Pradesh, India.

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The wild relatives of pigeonpea [*Cajanus cajan* (L.) Millsp.] are important source of genetic variation carrying genes for resistance to various biotic and abiotic stresses and other morphological traits. In the present study, four wild relatives of pigeonpea were evaluated using 24 simple sequence repeat (SSR) markers to assess their genetic diversity at molecular level. Each marker, on average, amplified 3.3 alleles with polymorphic information content (PIC) value of 0.53. The dendrogram pattern revealed two distinct genotypic clusters and cultivated pigeonpea was closely related to *Cajanus cajanifolius*. On the contrary, *Cajanus scarabaeoides* was the most diverse from the cultivated type. The results also suggest that genetic distance between cultivated pigeonpea and wild species was not related to their hybridization barrier.

Key words: *Cajanus*, crossability, genetic diversity, simple sequence repeat markers, wild relatives.

INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is an important legume cultivated in the tropics and sub-tropics for its high protein (18 to 22%) seeds. It is known to enrich soil through nitrogen fixation, release of phosphorous, and by adding valuable organic matter and micronutrients (Saxena, 2008). In pigeonpea germplasm, a large phenotypic variation exists for most economically important qualitative and quantitative traits (Remanandan, 1990). This variability, however, was not large enough to help breeders in developing high yielding cultivars and it resulted in stagnation of yield for over the past 50 years (FAO, 2010). Based on these observations, concerns have been expressed about the presence of useful genetic variability in the primary gene pool of pigeonpea. Under this scenario, while breeding cultivars, it is logical to use genetic variation available in the related wild species. To break this yield barrier through hybrid technology in pigeonpea, Saxena et al. (2005) developed a cytoplasmic nuclear male-sterility system and the wild relatives of pigeonpea played a major role in the development of diverse male sterility systems (Saxena et al., 2010a). This paper reports molecular variability in *Cajanus scarabaeoides*, *Cajanus cajanifolius* and *Cajanus acutifolius* representing secondary gene pool while *Cajanus platycarpus* selected from tertiary gene pool.

According to van der Maesen (1990), genus *Cajanus* comprises of 32 species, and of these, only *Cajanus cajan* is cultivated. These species are primarily distributed in India and Australia and have diploid chromosome 2n = 22. The success rate of hybridization of wild relatives with cultivated type was considered as an indicator of genetic diversity and the distantly related species do not cross easily to the cultivated type (Harlan and de Wet, 1971). Based on cyto-taxonomic studies, van der Maesen (1986) reported that *C. cajanifolius* of secondary gene pool was closest to the cultivated type and on the contrary, *C. platycarpus* was most diverse species and it was classified in tertiary gene pool. The other two species

*Corresponding author. E-mail: bharati_bth@yahoo.com. Tel: 04030713351. Fax: 30713074.

Table 1. Characteristics of the wild accessions used in the genetic diversity study.

Species	Wild accession character				
	ICPW 9	**ICPW 94**	**ICPW 29**	**ICPW 68**	**ICPL 87119**
	Cajanus acutifolius	***Cajanus scarabaeoides***	***Cajanus cajanifolius***	***Cajanus platycarpus***	***Cajanus cajan***
Growth Habit	Erect shrub with branches covered by short slivery hairs	Trailing bush with slender soft hairy stems	Erect and tall perennial shrub	Herbaceous twining bushy plant with slender hairy stems	Erect shrub with green stem
Leaflets	Dull green densely covered by velvety hairs and numerous resin glands	Leathery with dense white pubescence on low surface	Glandular punctuate with white pubescence on lower surface	Membraneous and faintly dotted	Glandular punctuate with dense hairs along the veins
Flowers	Yellow with reddish brown and purplish streaks	Yellow with dorsal red veins	Yellow with dorsal red veins	Pale Yellow	Yellow with red streaks on the back of standard petal
Pods	Oblong with 2-4 seeds	Wide flattened with 3-5 seeds	Oblong with 3-4 seeds	Flat-oblong with 2-4 seeds	Oblong with 2-5 seeds
Seeds	Oblong with brown and black color mosaic	Rectangular grayish with black and cream color mosaic	Round, black colored with grey mosaic	Wide rectangular and brown	Round and brown colored
Strophiole	Prominent	Prominent	Large	Large	Vestigial
Location collected	Australia	Sri Lanka	India	India	India

were placed somewhere in between the two. Yang et al. (2006) evaluated 322 pigeonpea accessions and reported that there was no relationship between phenotypic and molecular diversity. However, greater understanding of molecular diversity in genus *Cajanus* may help in enhancing productivity of inbred cultivars. This information may also be useful in breeding potential parental materials for enhancing the realized heterosis for seed yield.

Among the different markers generated in pigeonpea, simple sequence repeat (SSR) markers are available in reasonably good numbers which could be used to study in genetic diversity and their co-dominant nature make them the markers of choice for molecular breeding. Therefore, to generate information on this aspect, the present study was undertaken to assess the molecular diversity among wild relatives of pigeonpea using SSR markers.

MATERIALS AND METHODS

Four wild relatives of pigeonpea representing secondary (*C. acutifolius*, *C. scarabaeoides*, *C. cajanifolius*) and tertiary (*C. platycarpus*) gene pools were used in this study along with a popular *C. cajan* cultivar ICPL 87119. One accession of each species was selected randomly for genetic analysis and their important morphological traits are described in Table 1.

DNA extraction, SSR markers, polymerase chain reactions (PCRs) and electrophoresis

Seeds of the four wild and a cultivated species were sown in plastic pots and placed in a glasshouse. Three week old plants were used to extract DNA and it was purified using the protocol described in Cuc et al. (2008). The DNA quantity was assessed on 0.8% agarose gel and the DNA concentrations were normalized to 5 ng/µl. A total of 24 unlabeled primer pairs (Bohra et al., 2011) were used for molecular characterization of each genotype. PCRs were performed in a 5 µl reaction volume [0.5 µl of 10 × PCR buffer, 7.5 mM of $MgCl_2$, 1 mM of dNTPs, 1.5 pM of primer (MWG-Biotech AG, Bangalore, India), 0.3 U of Taq polymerase (Bioline, London, UK) and 5 ng of template DNA] in 96-well microtitre plate (ABgene, Rockford, Illinois, USA) using thermal cycler Gene-Amp PCR System 9700 (Applied Biosystems, Foster City, California, USA). A touch-down PCR

Table 2. Polymorphism among four wild relatives and one cultivated pigeonpea species.

SSR marker	Number of alleles	PIC	
CcM2409	2	0.27	
CcM2505	2	0.27	
CcM0785	2	0.27	Less polymorphic markers
CcM1982	2	0.27	
CcM1079	2	0.27	
CcM2332	2	0.36	
CcM0594	3	0.50	
CcM0988	3	0.50	
CcM1109	3	0.50	
CcM1373	3	0.50	
CcM1366	3	0.50	
CcM0673	3	0.50	
CcM2895	3	0.50	
CcM1011	3	0.50	
CcM2221	3	0.56	
CcM2697	4	0.67	High polymorphic markers
CcM2379	4	0.67	
CcM1207	4	0.67	
CcM2871	4	0.67	
CcM0962	4	0.67	
CcM2710	4	0.67	
CcM2818	5	0.77	
CcM0443	5	0.77	
CcM2241	5	0.77	
Mean	3.3	0.53	

program was used to amplify DNA fragments. An initial denaturation for 3 min at 95°C was followed by initial 10 cycles of denaturation for 20 s at 94°C, annealing for 20 s at 55°C (the annealing temperature for each cycle being reduced by 1°C per cycle) and extension for 30 s at 72°C. Subsequently, 35 cycles of denaturation (20 s at 94°C), annealing (20 s at 48°C), and extension (30 s at 72°C) were used, followed by final extension at 72°C for 20 min. The PCR products were checked for amplification on 1.2% agarose gel.

The amplified products were separated on capillary electrophoresis using ABI 3730 (Applied Biosystems, Foster City, CA, USA) and allele calling was performed using GeneMapper software version 4.0 (Applied Biosystems, Foster City, CA, USA). The allelic data obtained in bp were analyzed as allele sizes. The allelic data was used to prepare dendrogram using MEGA version 5.05 (Tamura et al., 2011). The polymorphism information content (PIC) refers to the value of a marker for detecting polymorphism within a given germplasm, depending on the number of detectable alleles and the distribution of their frequency. PIC value of markers was calculated using the formula recommended by Anderson et al. (1993).

RESULTS

The mean PIC value of the markers among the test materials was 0.53 (Table 2). The 24 polymorphic markers amplified a total of 78 alleles with an average of 3.3 alleles per marker. The PIC value ranged from 0.27 (CcM2409, CcM2505, CcM0785, CcM1982, CcM1079) to 0.77 (CcM2818, CcM0443, CcM2241); while the number of alleles varied from two (for markers CcM0785, CcM1079, CcM1982, CcM2332, CcM2409, and CcM2505) to five (for markers CcM0443, CcM2241 and CcM2818). Out of 24 polymorphic markers, 18 showed PIC values ranging from 0.50 to 0.77. The markers CcM0443, CcM2241 and CcM2818 were highly polymorphic with a PIC value of 0.77. This set of markers displayed a considerable polymorphism and can be used in constructing a genetic map, trait mapping or diversity studies with larger set of genotypes. A perusal of dendrogram (Figure 1) revealed two distinct clusters, cluster I included cultivar ICPL 87119 while cluster II contained all the wild relatives. This clearly indicated significant diversity between the wild relatives and the cultivated type. Similar results were also reported by Kassa et al. (2012) using single nucleotide polymorphism (SNP) markers. Among the wild species, *C. cajanifolius* showed close association with the cultivated species and it confirmed the results of Saxena et al. (2010b) and Kassa et al. (2012). This information also supported the taxonomical (van der Maesen, 1980) and

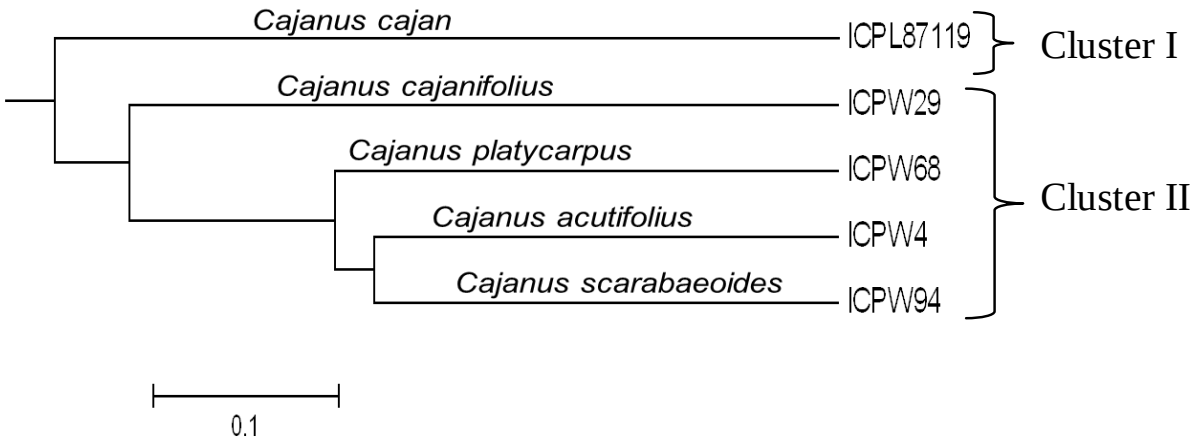

Figure 1. A dendrogram exhibiting molecular variation among four wild species and one cultivated species.

cytological (Mallikarjuna et al., 2012) findings reported earlier.

Based on the morphological similarities, De (1974) and van der Maesen (1980) postulated that the cultivated pigeonpea originated from its wild relative *C. cajanifolius* through macro-mutations. The present results also show that the molecular diversity among *C. scarabaeoides*, *C. acutifolius* and *C. platycarpus* was not large and they were grouped together in cluster II.

DISCUSSION

This study generates valuable information on the relationship between molecular diversity and crossability within genus *Cajanus*. Earlier, it was believed that *C. platycarpus* cannot be crossed with the cultivated type using normal procedures, as it is very diverse in comparison to other species; and hence, it was classified in the tertiary gene pool (Harlan and de Wet, 1971). Our studies, on the contrary, found that the genetic diversity of *C. platycarpus* was comparable with those of *C. scarabaeoides* and *C. acutifolius*, which can easily be crossed with the cultivated type, and thus were placed in the secondary gene pool. Therefore, it can be inferred that hybridization success may not always be a reliable criterion to predict the genetic diversity. Genetic diversity is known to play an important role in the genetic enhancement of the crop. In general, the crop wild relatives are rich source of useful genes which could be utilized in the development of novel plant types through hybridization and selection. In pigeonpea breeding program, some crossable wild species have been successfully used to incorporate traits such as high protein, earliness, and cytoplasmic nuclear male-sterility from C*. scarabaeoides* (Saxena et al., 1990; Saxena and Kumar, 2003) and pod borer resistance from *C. acutifolius* (Mallikarjuna and Saxena, 2002). *C. platycarpus* is known to harbor useful genes for traits like early flowering and maturity, photoperiod insensitivity, prolific flowering and pod setting, salinity tolerance, resistance to *Phytophthora* blight and *Helicoverpa* pod borer (Mallikarjuna et al., 2006).

Hybridization between *C. cajan* and *C. platycarpus* using normal procedures has repeatedly failed. The first success in hybridizing these two species was reported by Mallikarjuna and Moss (1995), who adopted embryo rescue technique to produce the inter-specific hybrid plants. They also reported that the natural hybridization barrier between the two species was post-zygotic in nature and it could be overcome by the application of gibberlic acid to allow initial development of embryo before excising it for growing in artificial media. Thus, it can be inferred that *C. platycarpus* carries one or few additional major gene(s) which inhibits the growth of zygote; and it is reflected as a strong hybridization barrier. Ahmad et al. (1992) also reported similar results in chickpea (*Cicer arietinum*) and concluded that evolution of reproductive barrier between the cultivated and wild species was not related to their genetic divergence. Thus, the genetic variability in the four wild species used in this study could be utilized in the development of new genotypes with traits like dwarfs, high protein (28 to 31%), CMS systems, cleistogamy, photoperiod insensitivity, prolific flowering and pod setting, and resistance to various biotic and abiotic stresses (Saxena et al., 1992, 2002, 2010a; Saxena and Sharma, 1995; Mallikarjuna et al., 2006).

The availability of large scale genomics resources and wide hybridization can be used to exploit potential of the wild relatives by detection and transfer of desirable genes through targeted genetic enhancement programs. The genetic diversity in the wild gene pool could also be used in breeding new inbred parents with desirable characteristics for increased hybrid vigor. From the present studies, it is concluded that: (i) a considerable genetic variation exists among the wild relatives of pigeonpea and, (ii) genetic distance among these species was not related to their hybridization barrier. The logical next step should be to study the intra-species molecular diversity of

these wild species and choose the most diverse accessions for breeding of high yielding hybrid/pure line cultivars.

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