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Efficiency of three DNA markers in revealing genetic variation among wild *Cajanus* species

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Abstract

Wild relatives of pigeonpea (*Cajanus cajan* L.) possess many useful genes that can be utilized for crop improvement, most importantly genes for resistance to *Helicoverpa armigera*, the legume pod borer. The present study aimed at quantifying diversity in a collection of *Cajanus scarabaeoides, Cajanus sericeus, Cajanus reticulatus* and *C. cajan* species selected from a wide geographic range using two PCR-based marker systems, amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSRs), and the hybridization-based restriction fragment length polymorphism (RFLP). Polymorphism was higher among the wild accessions than among the cultivated genotypes. Wild and cultivated *Cajanus* accessions belonging to different species clustered into four distinct major groups largely based on the interspecific differences. *C. scarabaeoides* accessions derived from same geographical origins formed one group reflecting similar genetic makeup of these accessions. Dendrograms generated using AFLP, RFLP and SSR marker data were comparable with minor clustering differences, which suggests that either method, or a combination of both can be applied to expanded genetic studies in *Cajanus*. Mantel testing confirmed the congruence between the genetic distances of three markers, indicating that the markers segregated independently, giving similar grouping patterns of all accessions having similar genetic origin.

Keywords: AFLP; mantel test; MDS plots; principal coordinate analysis; RFLP; SSRs

Introduction

Pigeonpea (*Cajanus cajan* (L.) Millspaugh) ranks fifth in importance among legume crops (FAO, 2006) and is cultivated in the dry lands of South Asia, Africa and Latin America. Its seeds are rich in protein and form an important component of the vegetarian diet in South Asia. The crop also enriches soil nitrogen and provides animal fodder and fuel wood. Much progress has been made in developing pigeonpea lines with tolerance to biotic and abiotic stresses through conventional plant breeding and improved management practices. However, yields of pigeonpea in the farmer's fields have remained stagnant over the past four decades, largely due to insect pest damage – *Helicoverpa armigera* (Hubner) being one of the most important yield-reducing factors (Green *et al.*, 2002).

In the past, phenological and morphological characters have been used for the assessment of diversity among

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cultivated pigeonpea and their wild relatives. The advent of environmentally neutral molecular markers has allowed better quantification of genetic diversity (Clegg *et al.*, 1984; Gepts, 1995). These technologies include restriction fragment length polymorphisms (RFLP; Botstein and White, 1980; Rafalski and Vogel, 1996), random amplified polymorphic DNA markers (RAPD; Bowcock, 1994), amplified fragmented length polymorphisms (AFLP; Zabeau and Vos, 1993) and simple sequence repeats or microsatellites (SSRs; Tautz, 1989). RFLPs have been used to characterize the genetic diversity among some cultivated crop species and their wild relatives (Beckmann and Soller, 1983; Wang and Tanksley, 1992; Sivaramakrishnan *et al.*, 2001, 2002). SSRs or microsatellites are highly polymorphic and are turning out to be the marker of choice in both animal and plant species (Condit and Hubell, 1991; Akkaya *et al.*, 1992; Morgante and Oliveri, 1993).

Among the 271 accessions belonging to 47 wild species of *Cajanus*, available in the collection maintained at Rajendra S Paroda Genebank at ICRISAT, *Cajanus scarabaeoides* is the most widely distributed. *C. scarabaeoides* can be easily crossed with cultivated pigeonpea and thus any of its useful genes can be utilized for the improvement of the latter. *Cajanus sericeus* and *Cajanus reticulatus* also posses certain useful genes that can be used in the genetic improvement of cultivated pigeonpea (Remanandan, 1988). Van der Maesen (1986,1990) produced a morpho-taxonomical description of the species but there are no published reports on the variation within the species for economic traits and only limited assessments at the molecular level (Nadimpalli *et al.*, 1993; Ratnaparkhe *et al.*, 1995; Sivaramakrishnan *et al.*, 2001, 2002). The present study aimed to assess the intraspecific diversity at molecular level between *C. scarabaeoides* accessions using different molecular markers. In addition, the interspecific variation among the four species (*C. scarabaeoides*, *C. sericeus*, *C. reticulatus* and *C. cajan*) was assessed using RFLP of mtDNA, AFLP and SSR markers.

Materials and methods

Plant material and DNA extraction

The 42 accessions used included 31 *C. scarabaeoides* from six countries (India, Sri Lanka, Australia, Philippines, Indonesia and Myanmar), four *C. sericeus* from India and Australia, one *C. reticulatus* from Australia and six *C. cajan* (Table 1). The two other wild species were included to sample interspecific variation among wild species. There are only four accessions each of *C. sericeus* and *C. reticulatus* in the ICRISAT genebank. Although all four accessions of each species were included, repeated attempts to extract DNA from three of the *C. reticulatus* accessions failed. The six pigeonpea accessions are high-yielding cultivars, but are susceptible to pod borer. Plants were grown in the glass house and DNA was extracted with the CTAB method (Murray and Thompson, 1980) from 5 g of young leaf collected from ten 1-month-old plants per accession.

Molecular marker diversity

Molecular marker diversity was assessed among the wild and cultivated pigeonpea using AFLPs, SSRs and RFLP.

RFLP analysis

About 15 μg DNA was digested with three restriction enzymes (*Eco* RI, *Hin*dIII and *Eco* RV; Amersham Pharmacia, Newcastle upon Tyne, UK), as per the manufacturer's protocols. Probes (the maize mitochondrial sequence

Table 1. Polymorphism and range for band size in wild and cultivated pigeonpea genotypes with maize mt probes

Enzyme–probe	No. of bands	No. of polymorphic bands	Diversity index	Effective multiplex ratio	Marker index	Size (Kb) of bands
Eco R1-*atp*6	14	14	0.88	14.00	12.32	2.1 to 23.1
Hind 111 – *atp* 6	10	9	0.89	8.10	7.21	2.0 to 9.2
Eco R V – *atp*6	6	5	0.84	4.17	3.50	3.0 to 14.8
Eco R 1–*atp* α	13	13	0.82	13.00	10.66	2.0 to 15.8
Hind 111 – *atp* α	10	10	0.87	10.00	8.70	7.7 to 14.4
Eco R V – *atp* α	8	7	0.82	6.13	5.03	2.1 to 11.2
Eco R 1-*cox* 1	11	11	0.94	11.00	10.34	4.1 to 14.2
Hind 111 – *cox* 1	9	8	0.81	7.11	5.76	3.4 to 11.6
Eco R V – *cox* 1	5	5	0.82	5.00	4.10	2.0 to 11.2
Mean	8.65 ± 0.987	8.20 ± 1.070	0.85 ± 0.050	8.73 ± 1.16	7.51 ± 1.050	
Polymorphism (%)	95.34%					

Bootstrap value (0.85–0.96) at 95% confidence level.

atp 6 (Dewey *et al*., 1985), *cox* I and *atp* α (Isaac *et al*., 1985) were ^{32}P labelled by random priming (Feinburg and Vogelstein, 1983), and the RFLP procedure followed Sivaramakrishnan *et al*. (2001).

AFLP analysis

AFLP analysis was carried out using a commercial kit (Life Technologies, Gaithersburg, MD, USA) following the manufacturer's protocols. Three *Eco*RI (+3) and three *Mse*I (+3) primers were used in five combinations.

SSR analysis

Ten SSR primer pairs (Burns *et al*., 2001) were used for genotyping. Each 25 μl reaction contained 25 ng of genomic DNA, 1 × PCR buffer (50 mM KCl, 20 mM Tris–HCl (pH 8.4)), 10 pmol of each primer, 2 mM $MgCl_2$, 200 nM dNTP, 50 μM dATP and 1U Taq DNA polymerase (Amersham Pharmacia). For labelling the PCR amplification products, 10 pmol of forward primer was radiolabelled by adding 1 μCi of [α^{32} P]-dATP to the reaction mix. The PCR programme was 94°C for 5 min, followed by 30 cycles of 94°C for 60 s, 55°C for 50 s, 72°C for 50 s, and ending with an extension step of 72°C for 5 min. PCR products were electrophoresed on a 6% denaturing polyacrylamide gel at 1500 V for 2 h.

Statistical analysis

For each accession, scoring for AFLP data was carried out according to Pangaluri *et al*. (2006), RFLP data according to Sivaramakrishnan *et al*. (2002) and SSR data according to Odeny *et al*. (2007). The data were analysed using NTSYS-Pc version 2.1 (Rohlf, 1997). Allele sharing (Ps; Bowcock, 1994) or the proportion of alleles shared between two accessions averaged over the loci was used as measure of similarity for all marker types. This corresponds to the simple matching coefficient (Sokal and Micherner, 1958) for the dominant marker (AFLP) and the Dice indices or Nei and Li coefficient (Nei and Li, 1979) for co-dominant markers (RFLP and SSR).

The distance matrix D generated using the genetic distance between individuals (u,v) was subjected to sequential agglomerative hierarchical cluster analysis using unweighted pair group method arithmetic average (UPGMA) (Rohlf, 1997) and the relationship between accessions was visualized as dendrograms. Differences between the dendrograms were tested by generating co-phenetic values for each dendrogram and the assembly of the co-phenetic matrix for each marker type. The Mantel correspondence test (Mantel, 1967) was used to compare the similarity matrices and the degree of congruence for each marker type. Gene diversity (Hj; Anderson *et al*., 1993), expected heterozygosity (Nei and Li, 1973) and the marker index (Powell *et al*., 1996) were calculated. The effective multiplex ratio (EMR = $n_p\beta$), where n_p is the number of polymorphic loci in the germplasm and β ($n_p/(n_p + n_{np})$) is the polymorphic fraction, was calculated. A principal coordinate analysis (PCoA) was carried out on the distance matrix and the distance matrix D to visualize the genetic interrelationships among the accessions in two-dimensional PCoA plots, with the resultant scores for the samples on the first two components plotted pairwise for each marker type. Multidimensional scaling (MDS) plots were constructed on the distance matrix D and the stress values (*s*) calculated.

Results

Molecular marker diversity

In the RFLP studies, each combination, except *Eco* RV – *atp* α and *Eco* RV – *cox* 1, was able to uniquely fingerprint all the 42 accessions. The former two combinations could not distinguish the accessions of *C. reticulatus* from *C. sericeus*. The *Eco* R1 – *atp* 6 combination generated a maximum number of 14 hybridization bands ranging from 2.1 to 23.1 Kb and *Eco* RV-*cox* 1 combination was least polymorphic yielding only five bands (2–11.2 Kb), respectively (Table 1). Number of unique banding patterns/haplotypes ranged from 8 in *Eco* RV – *atp* α to 10 in *Hind* III – *atp* 6 combination. *Eco* RV – *atp* 6, *Hind* III – *atp* 6 and *Hind* III – *cox* 1 were the three combinations in which none of the 6, 10 and 9 patterns generated and were shared between any of the genotypes (Table S2). Details of EMR, MI and Hav as revealed from RFLP studies are given in Table 1. Pairwise similarities (Sij) among the *C. scarabaeoides* accessions ranged from 0.52 to 1.00 with an average of 0.71 ± 0.21.

In the AFLP analysis, a total of 447 scorable bands with five primer pairs were detected across 42 accessions. Table 2 gives details of the EMR, HI and Hav. Pairwise similarity coefficient (Sij) for all the 42 accessions ranged from 0.24 to 1.00 with an average of 0.51 ± 0.26.

Ten SSR primer pairs were used to study the diversity, of which only eight primer pairs amplified the alleles in all the accessions. Higher polymorphism was observed among the *C. cajan* genotypes where all the eight primer pairs amplified the alleles, while among the wild species only seven out of the eight amplified the alleles. A total of 52 alleles were detected with an average allelic richness of 6.5 alleles per locus (Table 3). The number of alleles ranged from 3 for CCB4 to 14 for CCB1. Gene diversity was generally high, ranging from 0.62 to 0.92. When classified at the species level, the gene diversity was the highest for *C. cajan* (0.80) followed by *C. scarabaeoides* (0.71), *C. sericeus* (0.68) and *C. reticulatus* (0.41). Primer pairs CCB4 amplified only in *C. cajan*

Table 2. Polymorphism and gene diversity in wild and cultivated pigeonpea as revealed AFLP markers

Primer combination	Total no. of bands	No. of polymorphic bands	Fraction of polymorphic bands (β)	Diversity index (Hav)	Effective multiplex ratio (EMR)	Marker index (MI)
E-ACT M-CTC	129	121	0.94	0.77	113.74	87.58
E-AGG M- CAC	94	89	0.94	0.66	83.66	55.25
E-ACG M-CAT	69	67	0.97	0.83	55.61	46.17
E-ACG M-CTA	78	72	0.92	0.74	66.24	49.02
E-ACG M -CTT	77	75	0.97	0.74	72.75	53.84
Total	447	426			404.70	303.53
Mean	89.45	85.2	0.95	0.75	80.94	60.71

(3 alleles) and failed to amplify in all other species. Table 3 gives details of EMI, MI and Hav.

Among the three markers, 100% polymorphism was observed for SSR markers, followed by 95.4% of AFLPs and 95.3% of RFLPs, but the highest effective multiplex ratio of 80.94 and marker index value of 60.71 were observed for AFLPs but diversity index was maximum for SSRs (0.89; Table S3).

Interrelationships among accessions

UPGMA dendrogram of wild and cultivated pigeonpea accessions for the combined data from RFLP, SSR and AFLP markers is given in Fig. 1. Thirty one accessions of *C. scarabaeoides* formed one cluster. These accessions further sub-clustered based on the geographical regions, India, Sri Lanka, Australia, and Myanmar and Indonesia. Mantel's test confirmed the congruence between the AFLP, RFLP and SSR genetic distances with stress (*s*) values of 0.91, 0.86 and 0. 89, respectively. PCoA and MDS analysis grouped the different accessions according to species and subgrouped them based on the different geographical regions. *C. scarabaeoides* accessions of Indian origin (both early and medium duration flowering) formed one group, separate from those originating from Sri Lanka, Australia, Indonesia and Philippines. All accessions of *C. scarabaeoides* originating from Sri Lanka were grouped together and those from Australia were in a different cluster. *C. sericeus, C. reticulatus* and *C. cajan* formed three different groups with no specific sub-clusters. *C. sericeus* accessions clustered into two different groups, one subgroup of Indian origin and the other of Australian origin. *C. reticulatus* was placed between *C. cajan* and *C. sericeus* (Fig. 2).

Discussion

Molecular marker diversity

Marker diversity assessment using RFLP markers

The strong hybridization signals obtained with three maize mitochondrial DNA probes in all the 42 accessions

Table 3. Polymorphism and gene diversity in wild and cultivated pigeonpea as revealed by SSR markers

S. No	Locus name	No. of alleles	Fragment size in bp	Gene diversity (Hav)	Effective multiplex ratio	Marker index
1	CCB1	14	130–210 bp	0.89	14.00	12.46
2	CCB2	*	*	*	*	*
3	CCB3	*	*	*	*	*
4	CCB4**	3	190–221 bp	0.62	3.00	1.47
5	CCB5	8	160–220 bp	0.91	7.79	7.09
6	CCB6	4	180–260 bp	0.86	4.00	7.74
7	CCB7	5	140–220 bp	0.92	4.42	4.07
8	CCB8	3	130–150 bp	0.89	4.41	3.94
9	CCB9	5	155–180 bp	0.82	5.00	4.12
10	CCB10	10	140–220 bp	0.89	11.52	8.90
Mean	5.20 ± 1.36		0.85 ± 0.034	7.39 ± 1.378	6.39 ± 1.301	
Mean	5.20 ± 1.36		0.85 ± 0.034	7.39 ± 1.378	6.39 ± 1.301	
Polymorphism %	100					
Bootstrap-based on 95% confidence level (0.89–0.94).						

* No amplification. ** Amplified only in *Cajanus cajan* accessions.

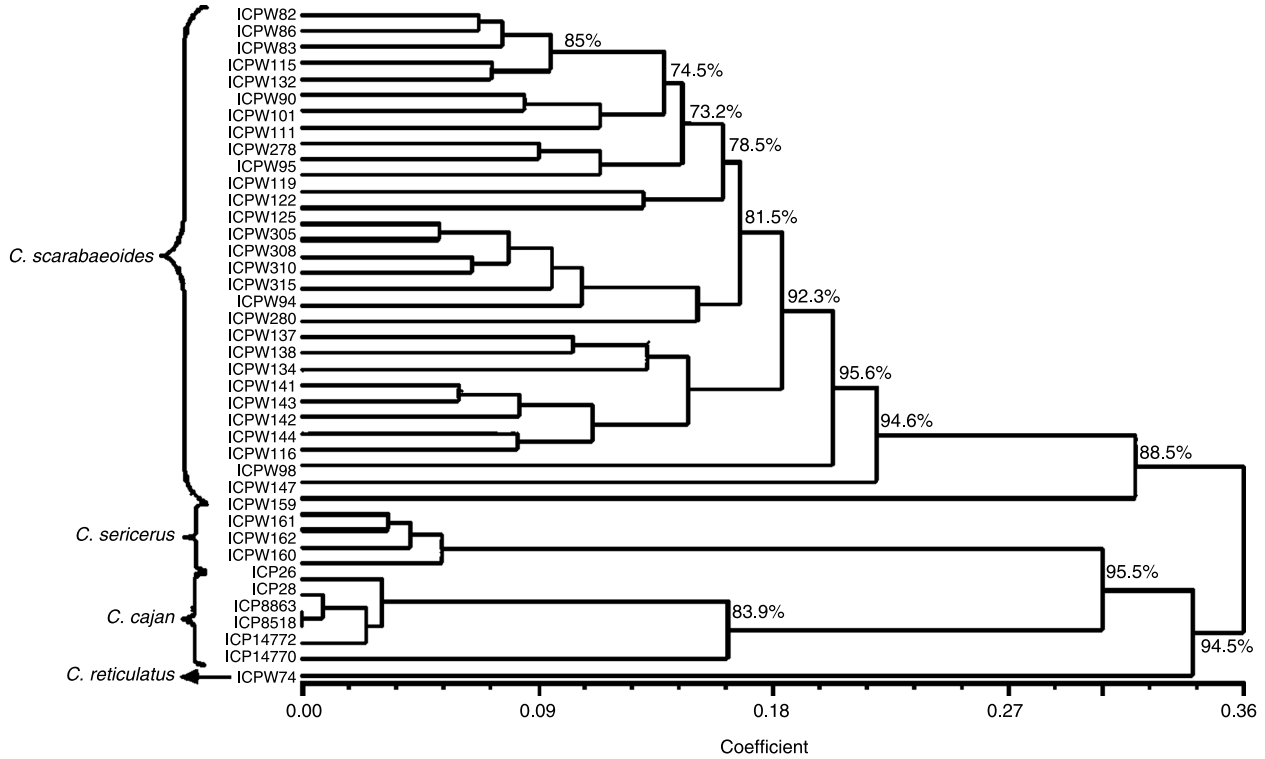

Fig. 1. UPGMA dendrogram of wild and cultivated pigeonpea accessions for combined data from three markers (AFLP, SSR and RFLP).

reflect high homology between the maize and pigeonpea mitochondrial DNA. Different sizes of bands were obtained with all the three multi-copy probes. Different relative intensities observed in some bands of *Eco* R1 – *atp* α and *Eco* R1 – *atp* 6 combination suggest variation in the copy number of these genes. Sivaramakrishnan (1999) and Sivaramakrishnan *et al.* (2001, 2002) in the assessment of genetic diversity with mitochondrial DNA probes among six wild *Cajanus* species observed similar results. Organelle genomes, such as mitochondrial genomes, were supposed to detect interspecific variations more efficiently than the intraspecific variations

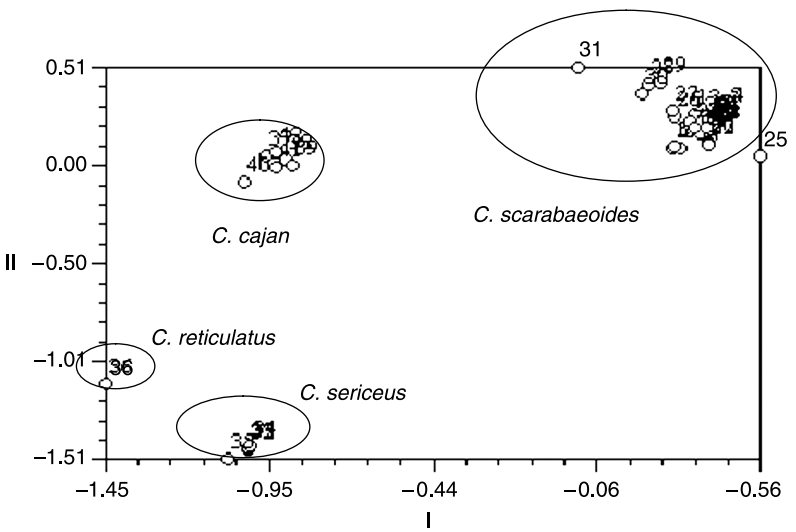

Fig. 2. Principal coordinate analysis plot based on the data from the three (SSR, AFLP and RFLP) markers. (Accessions list according to the Table 1).

(Ennos *et al.*, 1999). However, in the present study apart from the interspecific variation, the intraspecific variation was also detected efficiently as exemplified in *C. scarabaeoides*. Use of maize mtDNA probes for diversity analysis among the cultivated and wild accessions suggests the conserved nature of mitochondrial genome between cereals and legumes (Sivaramakrishnan *et al.*, 2001).

Marker diversity assessment using AFLP markers

AFLP studies revealed higher levels of polymorphism among the wild accessions, *C. scarabaeoides, C. sericeus* and *C. reticulatus* (97%) compared with the cultivated genotypes of *C. cajan* (27%). Lower levels of polymorphism in cultivated pigeonpea revealed by AFLP markers in the present study is in contrast with the high levels of polymorphism observed using AFLP markers in the cultivated species of barley, maize and pearl millet (Cervera *et al.*, 1998; Law *et al.*, 1998; Breyne *et al.*, 1999). In this study, the AFLP marker data revealed higher levels of genetic variation among the *C. scarabaeoides* accessions. Interestingly, the AFLP analysis indicated that accessions from different geographical locations with similar morphological characters such as days to flowering (Table S1) tend to cluster based upon their profiles, supporting the phenological classification of these accessions. One of the accessions, ICPW 147 (*C. scarabaeoides*, India), showed very unique AFLP banding pattern in all the primer combinations, and grouped separately from the other Indian accessions. Aruna *et al.*,(2005) reported that this accession has a unique mechanism (a combination of both antibiosis and antixenosis) of resistance to legume pod borer. Hence, the usefulness of AFLP marker in revealing the unique features of certain genotypes is particularly noteworthy and will be of immense use to pigeonpea breeders. The separation of *C. reticulatus* species from other wild species is in agreement with the fact that this wild species has distinct morphological and phenological characteristics distinct from other wild studied in the present investigation.

Molecular marker diversity using SSR markers

Seven out of ten microsatellites have amplified alleles in all accessions, of wild and cultivated, while two of the remaining three (CCB 2 and CCB 3) did not amplify alleles in any accessions, whereas CCB 4 amplified alleles in the cultivated genotypes. This might be because the microsatellites in *Cajanus* were designed based on the genome of cultivated accessions (Burns *et al.*, 2001). Though the SSR markers were limited in number, they were highly polymorphic and revealed maximum diversity index in bringing out the diversity among accessions. High diversity index obtained with SSRs is consistent with their known characteristics, that they are more variable, and provide higher resolution and higher expected heterozygosity that RFLPs, RAPDs or AFLPs (Powell *et al.*, 1996; Taramino and Tingey, 1996; Pejic *et al.*, 1998).

High levels of polymorphism associated with SSRs are expected because of the unique mechanism responsible for generating SSR allelic diversity by replication slippage (Tautz and Renz, 1984) rather than by simple mutations or insertions/deletions. Some SSR primer pairs (CCB 5 and CCB6) revealed higher levels of polymorphism within the cultivated types than the wild genotypes. The possibility of using SSR markers developed for one species in genetic evaluation of other species greatly reduces the cost of analysis (Moretzsohn *et al.*, 2004). SSR markers convincingly brought out differences between the early, medium and late flowering *C. scarabaeoides* accessions (Table S1) of Indian origin, further confirming the congruence with morphological/phenological grouping (Aruna *et al.*, 2004).

Comparison of the three molecular markers used in diversity analysis

This study clearly demonstrated that all the three marker types could be used for studying diversity among the wild and cultivated pigeonpea. All three marker types yielded highly polymorphic bands. Similar results were observed in wild and cultivated Sorghum (*Sorghum bicolor* L.) species (Kamala, 2003), where the levels of polymorphisms ranged from 60% for AFLPs, 80% for RFLPs and 100% for SSRs.

RFLP markers were found to be more efficient in bringing out the variation among the wild and cultivated species of pigeonpea in contrast to AFLPs, which could differentiate between wild accessions more efficiently. Although, AFLPs do not offer high levels of polymorphism, they have the capacity to reveal many polymorphic bands in a single lane. This is also consistent with the findings of other studies (Powell *et al.*, 1996; Russell *et al.*, 1997; Pejic *et al.*, 1998) and probably reflects two major differences between the genetic markers. First, as typically dominant markers, AFLPs ordinarily detect only two alleles per locus, which reduces the maximum possible levels of heterozygosity possible to 0.5. Second, SSR regions are well known to exhibit much higher levels of mutation than other parts of the genome (Jarne and Lagoda, 1996; Goldstein and Schlotterer, 1999).

The three molecular markers revealed closer proximity of *C. cajan* with *C. sericeus* and *C. scarabaeoides* compared with that of *C. reticulatus* (originated from Australia). In earlier studies of RFLP analysis of ribosomal DNA, Parani *et al.* (2000) had demonstrated that *C. reticulatus* was closer (95% similarity) to *C. platycarpus* (present in the tertiary gene pool) than to *C. scarabaeoides* (belonging to the primary gene

pool). A close genetic relationship between these two species (*C. scarabaeoides* and *C. cajan*) has also been reported earlier by seed electrophoresis (Krishna and Reddy, 1982; Kollipara *et al*., 1994). The present study also revealed closer relationship between *C. cajan* and *C. scarabaeoides,* which was also observed from the morpho-cytological, electrophoretic and molecular data (Pundir and Singh, 1985a; Nadimpalli *et al*., 1993; Ratnaparkhe *et al*., 1995). *C. scarabaeoides* is the most widely distributed wild species among all species of *Cajanus*, it has many important features like resistance to multiple disease and pest, high-protein content (Saxena *et al*., 1990) and its hybrids with *C. cajan* are highly fertile with normal meiosis (Pundir and Singh, 1985b; Van der Maesen, 1990).

The results of this study have added further information about the intra- and interspecific variation among the different *Cajanus* species that would be very useful to the plant breeders in exploiting the wild germplasm. This information can be used in breeding programmes and for the conservation and management of genetic resources. Despite the small number of SSR loci used in this study, the general congruence between the AFLP, RFLP and SSR datasets and their broad agreement with the morphological groups suggest that either molecular marker method or a combination of both is applicable to the expanded studies in the wild germplasm of *Cajanus*. The results obtained in the present study can be used to design breeding strategies to expand the genetic base of pigeonpea. However, a detailed study with more numbers of molecular markers (especially SSRs) conducted with a larger set of genotypes can be further useful to make better conclusions. The study of intraspecific variation with a larger dataset will help breeders exploit the diversity available particularly with in *C. scarabaeoides* genotypes. This is first study where intraspecific variation has been studied with multiple markers and hence the study can be further improved a lot to provide useful material for the breeders. Furthermore, if collections are available in genebanks from different parts of the world, they also should be included in such study for better conclusions. The results of this study, together with results of other morphological-, biochemical- and resistance-related characters might help in the selection of the most diverse parents for pod borer resistance-related characters and greatly expand genetic variation pigeonpea improvement.

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