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AFLP fingerprinting in pigeonpea (*Cajanus cajan* (L.) Millsp.) and its wild relatives

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Abstract

Detection of DNA polymorphism in cultivated pigeonpea (*Cajanus cajan*) and two of its wild relatives *Cajanus volubilis* and *Rhynchosia bracteata* is reported here for the first time using amplified fragment length polymorphism (AFLP) fingerprinting. For this purpose, two *Eco*RI (three selective nucleotides) and 14 *Mse*I (three selective nucleotides) primers were used. The two wild species shared only 7.15% bands with the pigeonpea cultivars, whereas 86.71% common bands were seen among cultivars. Similarly, 62.08% bands were polymorphic between *C. volubilis* and pigeonpea cultivars in comparison to 63.33% polymorphic bands between *R. bracteata* and pigeonpea cultivars, and 13.28% polymorphic bands among pigeonpea cultivars. The cluster analysis revealed low polymorphism among pigeonpea cultivars and very high polymorphism between cultivated pigeonpea and its wild relatives. The AFLP analysis also indicated that only one primer combination (*Eco*RI + ACT and *Mse*I + CTG), at the most any four primer pair combinations, are sufficient for obtaining reliable estimation of genetic diversity in closely related cultivars like pigeonpea material analyzed herein. AFLP analysis may prove to be a useful tool for molecular characterization of pigeonpea cultivars and its wild relatives and for possible use in genome mapping.

Introduction

Pigeonpea (*Cajanus cajan* (L.) Millsp.) is one of the major grain legume (pulse) crops of the tropics and subtropics. The Indian subcontinent, accounts for about 90% of the global production. Of the different pulse crops, pigeonpea ranks sixth in area and production but it is used in more diverse ways. Its seed protein content (approximately 21%) is also well comparable with that of other major grain legumes (Nene and Sheila 1990).

Wild relatives of pigeonpea serve as a rich source of disease resistance genes. For example, *Cajanus volubilis* (Blanco) Blanco has the elite character of resistance to sterility mosaic disease (Remanandan 1980). Similarly, *Rhynchosia bracteata* Benth. ex Bek possesses resistance to pod fly damage (Sharma et al. 2003). However, development of improved types through hybridization and recombination of available variability in pigeonpea and other species of *Cajanus* have met with only limited success (Saxena and Sharma 1990).

Attempts to improve production in both traditional and intensive production systems and to extend crop's adaptation beyond tropical and subtropical regions have recently gained attention. During the past three decades, breeders have developed a large number of short duration, large

seeded, high yielding types, stable for sole cropping under high levels of management, and disease resistant types in different maturity groups (Saxena and Sharma 1990). However, the initial limited success of an intensive effort does not indicate the ultimate efficiency of the breeding procedures, or full utilization of the genetic potential of the crop. Further research efforts need to concentrate on developing good understanding of genetic systems controlling qualitative and quantitative traits.

Knowledge about genetic diversity in available germplasm is very useful for plant breeders. It supports their decision on the selection of cross combinations from large sets of parent genotypes and is also helpful when they want to widen the genetic basis of a breeding program. Traditionally, morphological characters have been used for the identification of pigeonpea cultivars and its wild relatives, which necessitates growing the plants to full maturity prior to identification. Ladizinsky and Hamel (1980) used seed proteins electrophoresis to identify pigeonpea accessions, although very little polymorphism was detected. Later, restriction fragment length polymorphism (RFLP) technique was successfully used for detecting genetic diversity among the wild species of pigeonpea (Nadimpalli et al. 1992). Ratnaparkhe et al. (1995) have attempted randomly amplified polymorphic DNA (RAPD) to assess genetic diversity in pigeonpea and its wild relatives. Another type of markers, amplified fragment length polymorphism (AFLP) markers, have proved as more reliable and reproducible as compared to RAPD markers and less cumbersome and time consuming than the RFLPs. Paran et al. (1998) reported that although percentage of polymorphic bands was lower for AFLP than RAPD analysis, AFLP primers were more efficient in detecting polymorphism as AFLPs could detect polymorphism among closely related *Capsicum* cultivars that could not be detected by RAPDs. AFLP technique initially developed for fingerprinting plant genomes (Vos et al. 1995) has emerged as an important technique for genome mapping (Becker et al. 1995; Maheshwaran et al. 1997), gene tagging (Maksem et al. 1995), assessment of genetic diversity (Paul et al. 1997; Zhu et al.1998; Aggarwal et al. 2002; Bensnard et al. 2002), phylogenetic analysis of closely related plant species (Hill et al. 1996; Sharma et al. 1996; Aggarwal et al. 1999), and to assess somaclonal variation (Polanco and Ruiz 2002). In barley, Schut et al. (1997) have tried to associate the relationship measures based on AFLP markers, pedigree data and morphological traits to decrease the effect of their individual independent errors.

Here, we report for the first time, AFLP fingerprinting of some pigeonpea cultivars and two of its wild relatives to demonstrate the utility of this technique in assessing genetic diversity in this important pulse crop.

Materials and methods

Plant material

The plant material comprised of 14 Pusa cultivars and 6 ICPL cultivars of pigeonpea, and two wild species, *Cajanus volubilis* and *Cajanus bracteatus* (For details see Table 1). All the above material was obtained from The Pulse Research Laboratory, Indian Agricultural Research Institute, New Delhi, India.

DNA extraction

Genomic DNA was extracted from young leaves by CTAB method (Rogers and Bendich 1988) with a few modifications. The extracted DNA was purified by RNase treatment for 1–2 h at 37 °C and then with phenol–chloroform extraction. The pellet was dried and dissolved in appropriate volume of TE buffer (10 mM Tris, 1mM EDTA, pH 8).

DNA from different samples was quantified both by visual quantification (Agarose gel) and UV spectrophotometry.

AFLP reaction

AFLP assay was performed with AFLP analysis system-I (Invitrogen Life Technologies) as recommended by the manufacturers. Genomic DNA (250 ng) was digested with *Eco*RI and *Mse*I for 2 h at 37°C and the enzymes were inactivated at 70°C for 15 min. The DNA fragments were ligated with 24μL of *Eco*RI and *Mse*I adopter ligation mixture. For preselective amplification 5μL of 10-fold diluted ligation mixture was

Table 1. The pedigree and characteristics of pigeonpea cultivars and two of its wild relatives taken for AFLP analysis.

Serial number	Accession	Pedigree	Plant type	Seed size (g/100 seeds)	Characteristics
1	Pusa 2001	Progeny 27 cross no. 148 × upas 120	IDT	8.2	Medium tall, semi spreading, early maturity
2	Pusa 2008	Selection from ICPL 81	IDT	8.0	Medium tall, semi spreading, extra early in maturity
3	Pusa 951	EXN-5 × sel 90312	IDT	8.0	Tall, semi spreading, extra early in Maturity
4	Pusa 2003	Line 151 × Pusa 855	IDT	8.0	Medium tall, semi spreading, early maturity
5	Pusa 2001-6	Sel 90311 × Pusa 604	IDT	8.1	Medium tall, semi spreading, early maturity
6	Pusa 2001-2	ICPL154 × upas120	IDT	7.8	Tall semi, spreading extra, early maturity
7	Pusa 2001-3	EXN-5 × H88-25	IDT	8.2	Medium tall, semi spreading, early maturity
8	Pusa 991	Line 81 × sel 383		8.1	Tall, semi spreading, early maturity
9	Pusa 855	Mutant of T-21	IDT	9.0	Tall, semi spreading, early maturity
10	ICPL-182	C11 × ICP-1-6-W3 WB1	IDT	9.2	Short, semi spreading extra early maturity
11	Pusa 2002	P945 × Pusa 78	IDT	8.0	Tall, semi spreading, early maturity
12	Pusa 2001-1	Sel9-5 × sel 90309	IDT	7.6	Medium tall, bushy type, extra early maturity
13	Pusa 992	Sel from 90306 progeny-11		8.6	Medium tall, semi spreading, early in maturity
14	Pusa 2001-7	Sel 91031 × sel 90307	IDT	8.0	Tall, semi spreading, early in maturity
15	Pusa 2006	Pusa 604 × Pusa 78-1	DT	7.5	Medium tall, semi spreading, early in maturity
16	*C. volubilis*	Wild from Philippines or Indonesia	Climber		Climber on sal, teak or pine.
17	*R. bracteata*	Wild	Climber		
18	ICPL-11953	Germplasm line		8.5	Tall, semi spreading, late maturity
19	ICPL-11961	Germplasm line		6.6	Medium tall, semi spreading, early maturity
20	ICPL-87119	HY3C × PantA-2		11.2	Medium tall, semi spreading, early in maturity
21	ICPL-8858	Germplasm line		10.8	Medium tall, semi spreading, early in maturity
22	ICPL-11959	Germplasm line		7.0	Tall, semi spreading, late maturity

DT indicates determinant, IDT indicates Indeterminate. Extra early maturity is 120–140 days, early is 160 days and late maturity is above 200 days. Tall means above 2 m height, medium tall is 150–190 cm and short is up to 130 cm height.

amplified by 20 cycles of 94°C for 30 s, 56°C for 60 s, 72°C for 60 s using *Eco*RI and *Mse*I primers (each having one extra selective nucleotide) in a final volume of 50 μL.

For selective amplification, *Eco*RI primers with three selective nucleotides were labeled using T_4 polynucleotide kinase. ^{33}P labeled *Eco*RI (+3) primers (0.5μL) was mixed with 5μL of 50-fold-diluted preamplifier DNA, PCR buffer and the *Mse*I (+3) primers in a final volume of 20 μL. The reaction mixture was amplified for one cycle of 94°C for 30 s, 65°C for 30 s, 72°C for 60 s and subsequently lowering the annealing temperature by 0.7°C for each cycle for 12 cycles followed by 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. After completion of the cycle program, an equal volume of sequencing loading buffer [98% formamide (w/v), 10 mM EDTA, 0.25% xylene cyanol (w/v), 0.25% bromophenol blue (w/v)] was added to the reaction mixture. Prior to gel loading the mixture was heated for 3 min at 90°C and then immediately placed on ice.

Electrophoresis

The amplified fragments were analyzed on 6% denaturing polyacrylamide gel containing (20:1) acrylamide:bisacrylamid, urea (7.5 M) and 1×TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.3). For each gel, 100 mL casting solution was prepared and mixed with 200 μL 10% (w/v) ammonium persulphate and 30 μL TEMED. The gel solution was poured into 44.5 × 34.5 cm casting cassettes (Tharmo EC). Spacers and combs were 0.4 mm thick. TBE buffer was taken as electrophoresis buffer. Usually a 3 μL sample of each reaction mixture was loaded on the gel. Gels were run using a Tharmo EC (EC4000P) power pack at a constant power of 60 W and maximum voltage of 2000 V. Usually the gels were pre-electrophoresed at a constant power of 60 W for 30 min prior to sample loading. After electrophoresis, the gel was dismounted, taken on a Whatman paper sheet, wrapped with saran wrap and dried on a gel dryer. The dried gel was placed in a cassette and exposed to Kodak SB film at − 80°C overnight.

Data analysis

DNA fragment profiles representing a consensus of two independent replicates were scored in a binary mode with '0' indicating the absence and '1' indicating presence of band. Using the binary data, a similarity matrix was constructed using the Jaccard coefficient which was further subjected to UPGMA clustering analysis and a dendrogram was generated. A cophenetic matrix was constructed using the matrix that was used to generate the clusters. A correlation (mantel '*t*' test) between the cophenetic matrix and the similarity matrix was determined using MXCOMP module. All the above analysis was done using the software package NTSYS-PC (version 2.02e).

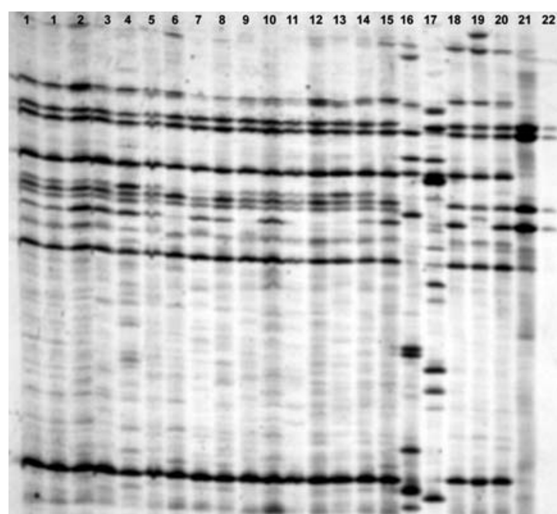

Figure 1. The autoradiogram of AFLP gel showing the pigeonpea cultivars and two wild relatives with the primer set *Eco*RI (+AAG) and *Mse*I (+CTG). Lane 1–15, pigeonpea cultivars Pusa 2001, Pusa 2008, Pusa 951, Pusa 2003, Pusa 2001-6, Pusa 2001-2, Pusa 2001-3, Pusa 991, Pusa 855, ICPL-182, Pusa 2002, Pusa 2001-1, Pusa 992, Pusa 2001-7, Pusa 2006; Lane 16, *Cajanus volubilis*; Lane 17: *Cajanus bracteatus*; Lane 18–22, pigeonpea cultivars ICPL-11953, ICPL-11961, ICPL-87119, ICPL-8858, ICPL-11959.

Results and discussion

In the present study on pigeonpea, we have analyzed 14 Pusa cultivars, 6 ICPL lines and two of its wild relatives using the AFLP fingerprinting approach.

Identification of pigeonpea cultivars

AFLP analysis revealed a large number of distinct scorable fragments per primer pair (Figure 1). A total of two *Eco*RI (with three selective nucleotides) and 14 *Mse*I (with three selective nucleotides) primers were used to amplify DNA from different accessions of pigeonpea. Out of these combinations, *Eco*RI (+ACT) + *Mse*I (+CAG) set shows monomorphic pattern in all these cultivars, while other combinations like *Eco*RI (+AAG) with *Mse*I (+CAG), (+CAC), (+CAT), (+CTA), (+CTG) and (+CTC), *Eco*RI (+ACT) with *Mse*I (+CAA), (+CAC), (+CAT), (+CTA), (+CTG), (+CTC) and (+CTT) were found to be promising in detecting polymorphism.

The number of polymorphic bands among different cultivars for each primer pair ranged from 1 to 36 (Table 2). Since AFLP markers are dominant, a locus was considered to be polymorphic if the presence or absence of the band was observed in various cultivars and monomorphic if the band is present among all the cultivars. The primer set *Eco*RI (+AAG) + *Mse*I (+CTA) yielded maximum number of polymorphic bands (36). Only two lines, namely ICPL-8858 and ICPL-11959, were found to be monomorphic. The primer sets *Eco*RI (+AAG) + *Mse*I (+CAC), *Eco*RI (+ACT) + *Mse*I (+CAA) showed the fewest polymorphic bands (one), in which all the five ICPL lines except ICPL-182 showed only monomorphic bands. Similarly, very low polymorphism was observed between the Pusa cultivars with all the primer sets except *Eco*RI (+AAG) with *Mse*I (+CTG), *Eco*RI (+AAG) with *Mse*I (+CAT) and *Eco*RI (+ACT) with *Mse*I (+CAC). Till date there is a solitary report available on the identification of pigeonpea cultivars at the DNA level (Ratnaparkhe et al. 1995). As a result, pigeonpea breeding relies heavily on phenotypic selection methods. Moreover, pigeonpea is one of the exceptions among the grain legumes in that though it is a predominantly self-pollinating crop, out-crossing level also varies widely (Saxena and Sharma 1990). As a result of frequent out-crossing, existing standard cultivars have become heterogeneous for several important agronomic characters such as disease resistance, maturity time, etc. The maintenance of germplasm in pigeonpea is very

Table 2. The number of polymorphic bands in *Cajanus volubilis, R. bracteata* and with in pigeonpea cultivars with different combinations of *EcoR*I (three selective nucleotides) and *Mse*I (three selective nucleotides) that are taken for AFLP analysis.

*EcoR*I primer selective nucleotides	*Mse*I primer selective nucleotides	Polymorphic bands in *C. volubilis*	Polymorphic bands in *R. bracteata*	Polymorphic bands within pigeonpea cultivars
+ AAG	+ CAG	60	70	7
	+ CAC	75	77	1
	+ CAT	69	70	2
	+ CTA	68	80	36
	+ CTG	66	59	36
	+ CTC	47	51	7
+ ACT	+ CAA	81	66	1
	+ CAG	37	43	0
	+ CAC	41	49	10
	+ CAT	40	50	6
	+ CTA	58	70	7
	+ CTG	67	70	20
	+ CTC	38	47	12
	+ CTT	45	58	3

tedious, and problems of contamination have been enormous. The molecular identification of cultivars will, therefore, be helpful in assessing the purity and stability of the genotypes entering into the breeding programs.

Genetic relationships within Cajanus cajan

In order to quantify the level of polymorphism detected by AFLP fingerprinting, Nei's estimate of similarity based on the probability that an amplified fragment from one genotype will also be found in another was used to generate a similarity matrix (Nei and Li 1979). All the accessions fell in the range of 0.82–1.0 (86.71% of common bands). This indicates little polymorphism (13.28%) at the DNA level between various accessions and may be due to their predominantly self-pollinating nature of the cultivars that were taken here. Other self-pollinated species such as tomato and wheat also show little polymorphism among accessions (Joshi and Nguyen 1993; Williams and Clair 1993). At present, there is little information available about genetic diversity among pigeonpea cultivars. Previously protein and isozyme electrophoresis were used to estimate variability in pigeonpea cultivars (Ladizinsky and Hamel 1980; Kollipara et al. 1994). The major limitation of these techniques is an insufficient number of polymorphisms detected among closely related cultivars. Earlier, RAPD markers were used to detect the genetic variability among pigeonpea cultivars (Ratnaparkhe et al. 1995). But AFLP markers are considered to be more reliable and reproducible as compared to RADP markers. Our data demonstrate that the AFLP technique can be applied for estimating the genetic variability among closely related cultivars.

The UPGMA dendrogram (Figure 2) analysis of pigeonpea cultivars shows three main clusters, which are further divided into subgroups. First cluster consists of 'ICPL-8858' and 'ICPL-11959', while the second cluster consists of 'ICPL-87119', 'ICPL-11961' and 'ICPL-11953'. Among them 'ICPL-11961' and 'ICPL-11953' form a subgroup and are more closely related to each other than to 'ICPL-87119'. The third cluster consists of six subgroups in which Pusa 2002 forms its independent subgroup as in case of Pusa 2001-3. The third subgroup has ICPL-182 and Pusa 855, which are very similar to each other. The fourth subgroup contains 'Pusa 2008', 'Pusa 2001-1' and 'Pusa 2001-2', out of which 'Pusa 2001-1' and 'Pusa 2001-2' are very similar to each other. The fifth subgroup contains Pusa 992, Pusa 2001-6, Pusa 2003, Pusa 991 and Pusa 951. Here Pusa 992 and Pusa 2001-6 are very similar to each other, and Pusa 991 and Pusa 951 are similar to each other leaving Pusa 2003 out. The sixth subgroup contains Pusa 2001-7, Pusa 2006 and Pusa 2001, in which Pusa 2006 and Pusa 2001 are more similar to each other than to Pusa 2001-7.

The clustering pattern generated through UPGMA analysis was validated by generating a cophenetic similarity value matrix from a set of nested clusters and comparing the cophenetic

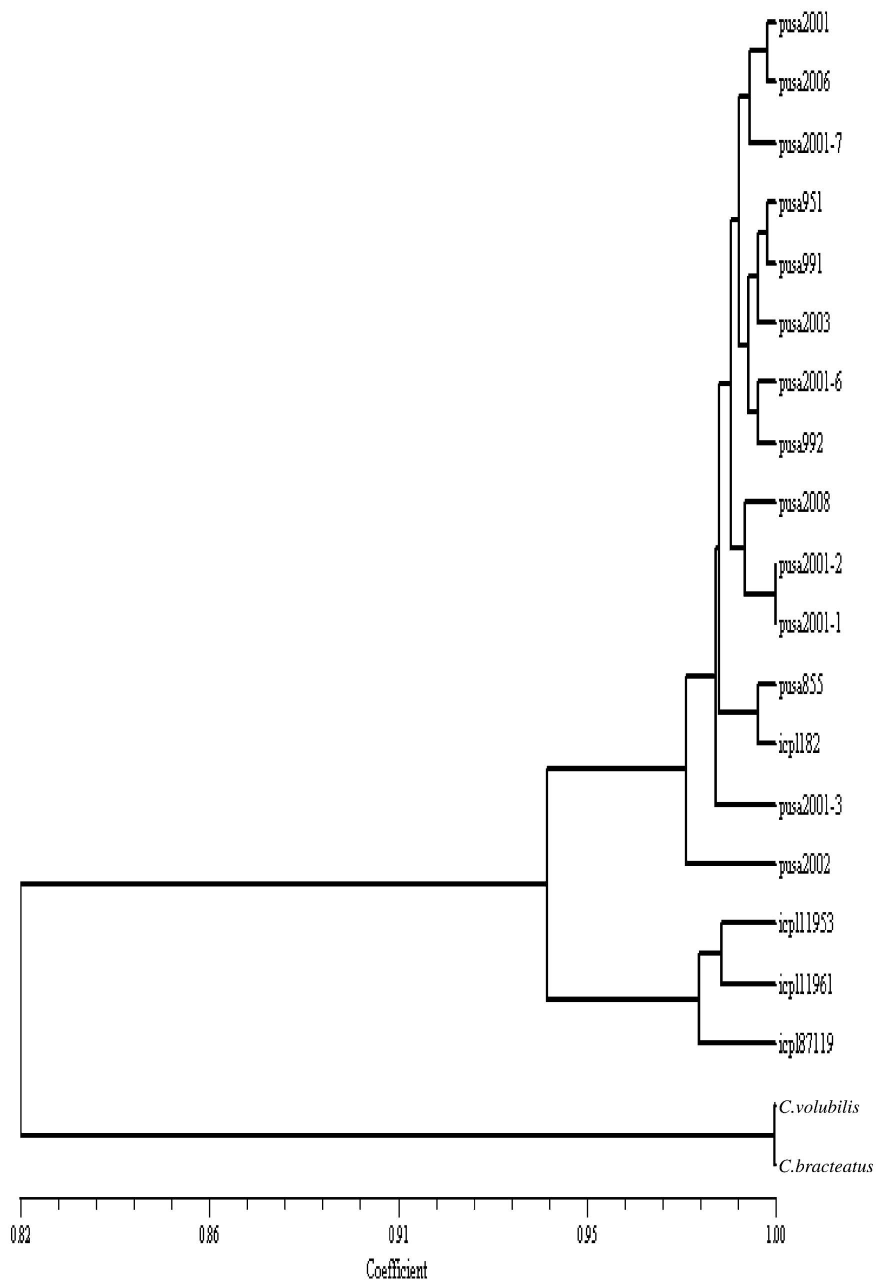

Figure 2. The UPGMA dendrogram of pigeonpea cultivars.

value matrix with the similarity matrix of the original data. The goodness of fit of the clusters tested using an MXCOMP module through a mantel '*t*' test, gave a very high correlation coefficient of 0.997, thereby indicating the validity of the clusters generated from the present study. The cophenetic correlation coefficient measures the agreement between the similarity values implied by the dendrogram and those of the original similarity matrix (Sneath and Sokal 1973).

Another important aspect of this investigation is to know the minimum possible number of AFLP markers needed for reliable grouping of the pigeonpea cultivars having closely related genetic background. Information on this line will help in reducing the labor and cost of an AFLP project.

For this purpose, we developed dendrograms from each primer pairs independently as well as from four primer pairs randomly in addition to the final dendrogram that was developed utilizing data from all the primer combinations. In this way, we found that the dendrograms developed from the primer pairs *Eco*RI (+ AAG) with *Mse*I (+ CTC) and *Eco*RI (+ ACT) with *Mse*I (+ CAC), respectively, have the same pattern with some minor differences. In these two dendrograms all the Pusa cultivars are grouped in a cluster leaving all ICPL cultivars as separate cluster with no subgrouping within the clusters. These primers can serve as good markers to distinguish the Pusa cultivars and ICPL cultivars from a pool. The dendrogram developed from the primer pair *Eco*RI (+ AAG) with *Mse*I (+ CTA) is almost similar to the final dendrogram that was developed from all the primer combinations. This clearly shows that single primer pair *Eco*RI (+ AAG) with *Mse*I (+ CTA) is sufficient for reliable grouping of closely related pigeonpea cultivars. The dendrograms that were developed from any four-primer pair combinations randomly also showed the same pattern with minor variations with the final dendrogram that was raised from all the primer pairs taken in this study. This clearly indicates that at the most any four of the tested primer pairs are sufficient for reliable estimation of genetic diversity in closely related cultivars like pigeonpea material analyzed herein. Recently, Aggarwal et al. (2002) have also reported a similar observation in rice.

We have also exploited the utility of RAPD markers (unpublished data) on these lines. The preliminary results established the utility of RAPD markers in assessing the genetic diversity in pigeonpea; however, the number of primers required to reveal a meaningful estimate of diversity is relatively high (~40).

Genetic relationship between wild species and cultivars

The wild relatives of pigeonpea viz; *Cajanus volubilis* and *Rhynchosia bracteatus* were selected to find the genetic relationships with the cultivars. Unlike in pigeonpea cultivars, extensive polymorphism was found between the two wild species and the cultivars. Many bands were common between the two wild species, even though few unique bands were also found. The percentage of polymorphism between *C. volubilis* and pigeonpea cultivars was found to be 62.08%, whereas between *R. bracteata* and pigeonpea cultivars it was 63.33%. The number of polymorphic bands between *C. volubilis* and pigeonpea cultivars varied from 37 to 81 for each primer set. Similarly, the number of polymorphic bands between *R. bracteata* and pigeonpea cultivars varied from 43 to 80 for each primer set (Table 2). From the UPGMA dendrogram based on wild species and pigeonpea cultivars (Figure 3), the similarity matrix between the wild species and cultivars ranged from 0.22 to 1.0, indicating a large amount of genetic variation between them. We also made an attempt to correlate the relationship measures based on AFLP markers, pedigree data and morphological traits in pigeonpea accessions. Due to insufficient morphological data and inclusion of some of the mutant genotypes (Pusa 855), some germplasm lines (ICPL-11953, 11961, 8858 and 11959) and wild species, we were unable to correlate these measurements with our fingerprint data.

Conclusions

The present investigation demonstrates the potential of AFLP fingerprinting in detecting polymorphism among pigeonpea cultivars, which have narrow genetic background particularly in case of Pusa lines. The AFLP analysis clearly indicated that only one primer pair *Eco*RI (+ AAG) with *Mse*I (+ CTA) or at the most any four primer pair combinations are sufficient for reliable estimation of genetic diversity, while with RAPD markers nearly 40 primers are required for the same in closely related cultivars like pigeonpea material analyzed herein. In addition to this, the markers generated via AFLP assay can provide practical information for the management of genetic resources. For the selection of good parental material for breeding program the genetic data produced through AFLP can be used to correlate with the relationship measures based on pedigree data and morphological traits to minimize the individual inaccuracies in pigeonpea. Further, a large amount of genetic variation exists between pigeonpea cultivars and its wild relatives, which can be used efficiently for gene tagging, and genome mapping of wild and cultivar crosses to

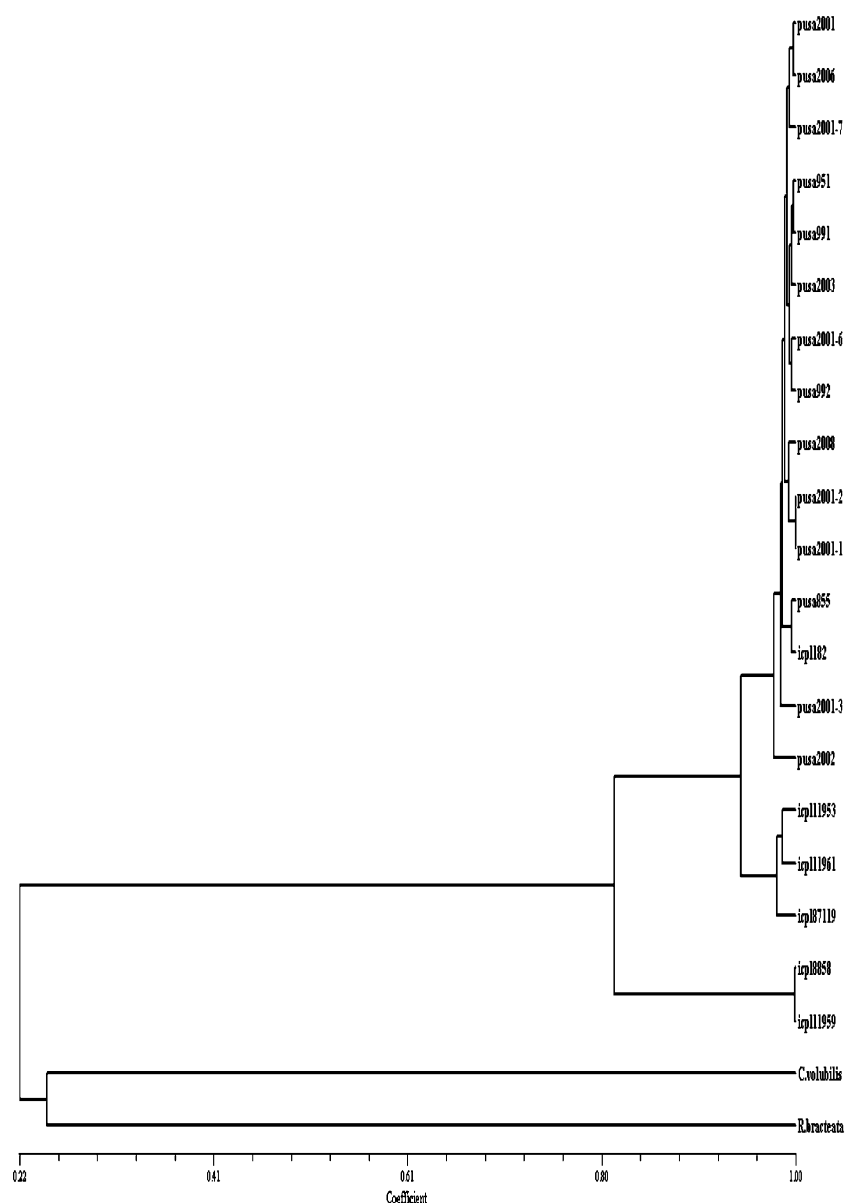

Figure 3. The UPGMA dendrogram of pigeonpea cultivars and two of its wild relatives.

introgress the disease and insect resistance into the cultivated genotypes.

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