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Genetic fingerprinting of pigeonpea [*Cajanus cajan* (L.) Millsp.] and its wild relatives using RAPD markers

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Abstract Randomly amplified polymorphic DNA (RAPD) markers were used for the identification of pigeonpea [*Cajanus cajan* (L.) Millsp.] cultivars and their related wild species. The use of single primers of arbitrary nucleotide sequence resulted in the selective amplification of DNA fragments that were unique to individual accessions. The level of polymorphism among the wild species was extremely high, while little polymorphism was detected within *Cajanus cajan* accessions. All of the cultivars and wild species under study could be easily distinguished with the help of different primers, thereby indicating the immense potential of RAPD in the genetic fingerprinting of pigeonpea. On the basis of our data the genetic relationship between pigeonpea cultivars and its wild species could be established.

Key words Pigeonpea · RAPD markers · Genetic relationship

Introduction

Pigeonpea is an important grain legume crop of the semi-arid tropics and is widely cultivated in the Indian subcontinent, Southeast Asia, Africa and Central America. The highest production of pigeonpea occurs in India, where it is the most widely grown legume after chickpea. Wild relatives of pigeonpea serve as a rich source of disease and insect resistance that can be introgressed into the cultivated genotypes. For example, wild pigeonpea sp. *C. volubilis* has been found to have the elite character of resistance to sterility mosaic diseases (Remanandan 1980). Pigeonpea is predominantly a self-pollinated crop, although outcrossing of up to 40% has been reported (Sen and Sur 1964). The outcrossing mechanism helps in the production of hybrid seed and in population improvement breeding schemes, but poses problems in the development of pure lines and in maintenance of the purity of released cultivars and the identification of the genetic purity of cultivars used in a specific breeding programme is of prime importance. Despite the major position of pigeonpea as an important legume crop, very little information about its genetics is available. Traditionally phenological and morphological characters have been used for the identification of pigeonpea cultivars and their wild relatives, however, these characters may not be significantly distinct and usually require growing the plants to full maturity prior to identification. Ladizinsky and Hamel (1980) used the electrophoresis of seed proteins to identify pigeonpea accessions, and very little polymorphism was detected among them. Recently restriction fragment length polymorphism (RFLP) markers have been successfully used for detecting genetic diversity among the wild species of pigeonpea (Nadimpalli et al. 1992). Randomly amplified polymorphic DNA (RAPD) markers provide yet another approach to identify and estimate genetic diversity among closely related cultivars and wild species. This method has been found to be of great use in constructing genetic linkage maps (Rafalski et al. 1991) and tagging genes such as downy mildew resistance in lettuce (Paran et al. 1991) and root knot nematode resistance in tomato (Williamson et al. 1994). Prior to the application of RAPD markers in various breeding programmes of pigeonpea, it is essential to show that RAPDs can decipher polymorphisms in pigeonpea genotypes. In the investigation presented here, we report the use of RAPD markers for the identification and estimation of genetic diversity in pigeonpea cultivars and their wild species.

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Materials and methods

Plant materials

The following pigeonpea cultivars were selected for polymerase chain reaction (PCR) analyses: 'PT-14', 'T-21', 'DPPA 85-12', 'ICPL 75-125', 'TAT-14', 'Bahar', 'MTH-12', 'ICPL-87', 'PT-31', and 'C-11'. The plant material was obtained from the Pulse Research Center, Mahatma Phule Agricultural University, Rahuri. The wild relatives of pigeonpea listed in Table 1 were obtained from International Crops Research Institute for the Semi-Arid Tropics, ICRISAT, Patancheru, India.

DNA extraction

Plant DNA was extracted from young leaves by the CTAB method (Rogers and Bendich 1988) with a few modifications. Young leaf tissue was ground in liquid nitrogen and mixed with 15 ml CTAB extraction buffer (2% hexadecyltrimethyl ammonium bromide, 100 m*M* Tris-HCl, pH 8.0, 20 m*M* EDTA, pH 8.0, 1.4 *M* NaCl, 1% polyvinyl pyrrolidone). The homogenate was then incubated at 60 °C for 15 min, emulsified with an equal volume of chloroform:isoamylalcohol mixture (24:1) and centrifuged at 10000 rpm. Equal volume of CTAB precipitation buffer (1% CTAB, 50 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, pH 8.0) was added to the supernatant, which was then mixed gently and centrifuged at 10 000 rpm. The pellet was dissolved in high salt TE buffer (1 *M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA) and was precipitated with twice its volume of chilled ethanol. The precipitate was washed with 70% ethanol, dried and redissolved in TE buffer. DNA was quantified in a fluorometer (Hoefer Instrument Model TKO-100) according to the instructions of the manufacturer.

PCR amplification

PCR amplification was performed as described by Williams et al. (1990) with a set of 20 oligonucleotides from Kit A (OPA1-OPA20) and 3 from kit B (OPB1-OPB3) synthesized by Operon Technologies (Almeda, Calif.). The reaction mixture (25 µl) contained 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% gelatin, 100 µ*M* of dATP, dTTP, dCTP and dGTP (Pharmacia), 5 pmoles of primers, 15 ng of genomic DNA and 0.3 U of *Taq* polymerase (Promega). Each reaction was overlaid with 100 µl of mineral oil to prevent evaporation. Amplification was carried out in a Perkin Elmer Cetus thermocycler for 45 cycles, each consisting of a denaturation step of 1 min at 94 °C, followed by an annealing step of 1 min at 36 °C and an extension step of 2 min at 72 °C using the fastest available temperature transitions. The last cycle was followed by 5 min at 72 °C to ensure that the primer extension reaction was completed. To reduce the possibility of cross contamination and variation in the amplification reactions, mastermixes of the reaction constituents were always used.

Agarose gel electrophoresis

The RAPD products (10 µl) were loaded on a 1.4% agarose gel for electrophoresis in 1 × TBE (89 m*M* Tris-HCl, pH 8.3, 89 m*M* boric acid, 5 m*M* EDTA) at 50 V for 6 h, stained with ethidium bromide and visualized by illumination under UV light.

Results and discussion

Optimization of amplification conditions

Assays to optimize the template concentration were conducted over the range of 0.5 to 200 ng. A constant band pattern was obtained at a template concentration between 5 and 30 ng. Ultimately, a template DNA concentration of 15 ng/25 µl was selected for the PCR amplification. Figure 1 shows a representative picture of PCR amplification using primer OPA1. The co-migra-

Table 2 Number of polymorphic bands among wild relatives of pigeonpea amplified by different primers

Primer	Number of polymorphic bands
OPA-1	12
OPA-3	11
OPA-4	8
OPA-5	13
OPA-6	14
OPA-7	22
OPA-8	25
OPA-10	18
OPA-11	15
OPA-12	16
OPA-13	12
OPA-15	21
OPA-16	25
OPA-17	10
OPA-18	11
OPA-20	14

Table 1 A list of species used in the present study

Serial no.	Species	Accession no.	Country of origin
1.	*Cajanus acutifolius*	IBS 2419	Australia
2.	*Cajanus albicans*	JM 3023	Karnataka, India
3.	*Cajanus goensis*	JM 3501	Kerala, India
4.	*Cajanus grandifolius*	PR 4256	Australia
5.	*Cajanus lineatus*	PR 5227	Kerala, India
6.	*Cajanus reticulatus*	IBS 2156	Australia
7.	*Cajanus sericeus*	JM 1961	Maharashtra, India
8.	*Cajanus volubilis*	JM 4208	Burma
9.	*Dunbaria ferruginea*	PR 5709	Tamilnadu, India
10.	*Rhynchosia rothii*	JM 3410	Tamilnadu, India
11.	*Rhynchosia bracteata*	JM 3952	Andhra Pradesh, India
12.	*Rhynchosia melacophylla*	ICPW 295	Unknown[a]
13.	*Flemingia stricta*	NKR 186	Andhra Pradesh, India
14.	*Cajanus cajan*	ICPL 8094	India
15.	*Cajanus cajan*	ICPL 317	India

[a] EC 130718 (source ICRISAT)

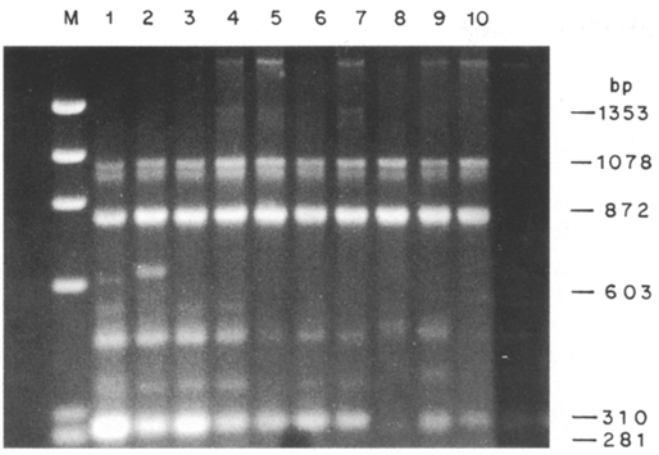


Fig. 1 Agarose gel electrophoretic pattern of PCR amplification products obtained with different pigeonpea cultivars using primer OPA1. *M* represents the phi X 174 *Hae*III marker. The sizes of the marker bands are given for comparison. *Lanes 1–10* pigeonpea cultivars 'PT-14', 'T-21', 'PT-31', 'DPPA 85-12', 'ICPL 75-125', 'TAT-14', 'Bahar', 'MTH-12', 'ICPL-87' and 'C-11'

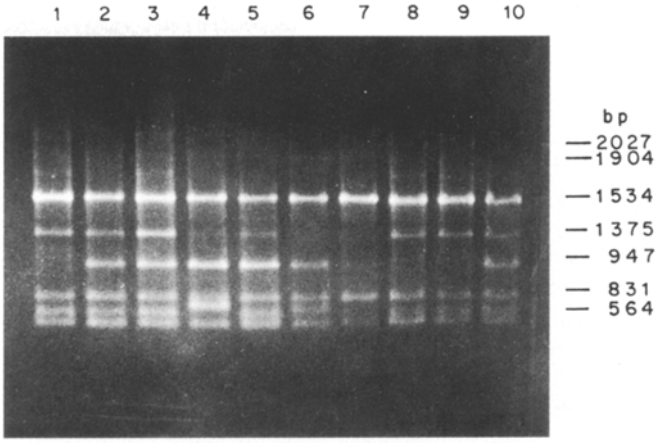


Fig. 2 Agarose gel electrophoretic pattern of PCR amplification products obtained with different pigeonpea cultivars using primer OPA13. *Numbers* at the *top of each lane* refer to the cultivars listed in Fig. 1

ting bands provided an internal control by which to monitor the reproducibility of the amplification patterns. Furthermore, a low magnesium concentration (1.5 m*M*) in the PCR reaction was found to be optimal for the purpose of producing clear and reproducible DNA fingerprints.

To determine the degree of heterogeneity within the population, we extracted DNA from different individuals of the same accessions and used this for PCR amplification. The RAPD pattern was found to be identical in all cases (data not shown). Successive analyses were performed routinely on DNA extracted from leaves pooled from at least ten individuals of the same accession.

Identification of pigeonpea cultivars

In an attempt to examine the potential of RAPD markers for their ability to identify pigeonpea cultivars, a set of 23 primers was used to amplify DNA from different accessions of pigeonpea. In total, 16 primers were found to be polymorphic, while 3 primers (OPA2, OPB1, OPB3) were monomorphic, and the other primers (OPA9, OPA14, OPA19, OPB2) did not show any amplification. The number of bands for each primer which produced a polymorphic band pattern varied from 3 (OPA6) to 11 (OPA18) in the size range of 0.25 to 2 kb. A total of 127 different RAPD products were amplified by 16 primers. Since RAPD markers are dominant, a locus was considered to be polymorphic if the presence and absence of the bands were observed in various individuals and monomorphic if the bands were present among all the individuals. Figure 2 shows a representative picture of the electrophoretic pattern of PCR-amplified DNA fragments of pigeonpea cultivars using primer OPA13. In the present study, no single primer was able to distinguish between all the cultivars. However, amplifications by different primers were informative and produced a cultivar-specific pattern. The marker is designated as the operon primer number, followed by molecular weight in base pairs of the amplified band. The markers OPA1-610, OPA2-1090, OPA6-872, OPA7-1904, OPA11-603, OPA12-2027, OPA12-1584, OPA15-603 and OPA17-400 were unique to different individuals. One fragment, OPA11-320, was unique to two cultivars, namely 'ICPL-87' and 'C-11'. Fragments OPA15-900 and OPA12-872 were specific to 'PT-31' and 'DPPA85-12'. Other fragments were present in more than two cultivars and were still polymorphic. The reproducibility of these results was evaluated by replicating the RAPD analysis on all the accessions with primer OPA11, OPA13 and OPA18.

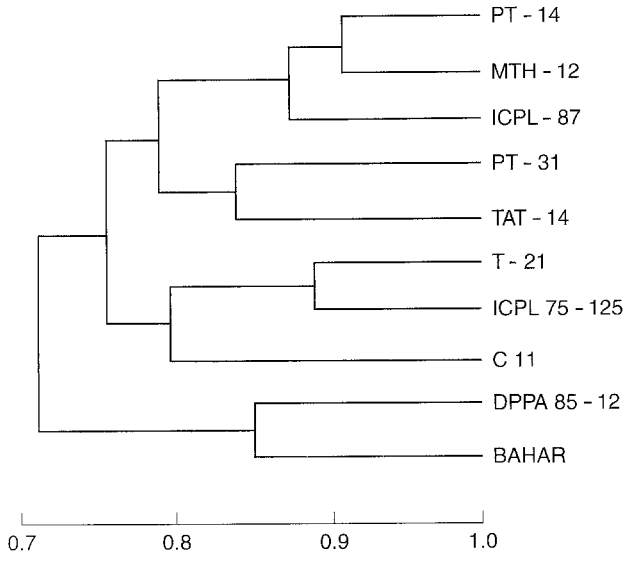
At present there is no information available on the identification of pigeonpea cultivars at the DNA level. As a result, pigeonpea breeding relies heavily on a phenotypic selection method. Secondly, pigeonpea is one of the exceptions among grain legumes in that it has a tendency towards outcrossing. As a result of frequent outcrossing, existing standard cultivars have become heterogenous for several important agronomic characters such as disease resistance. For instance, the cultivar 'Bahar' has lost its resistance to sterility mosaic disease, perhaps as a result of outcrossing with susceptible cultivars (Gupta et al. 1980). The maintenance of germplasm in pigeonpea is very tedious, and problems of contamination have been enormous. The identification of cultivars will, therefore, be helpful in assessing the purity and stability of the genotypes entering into the breeding programmes.

Genetic relationship within *Cajanus cajan*

In order to quantify the level of polymorphism detected, Nei's estimate of similarity, based on the probability that an amplified fragment from one plant will also be found in another, was used to generate a similarity matrix (Nei and Li 1979). All of the accessions fell in the range of 0.7 to 0.9. This indicates little polymorphism at the DNA level among various accessions and may be due to the predominantly self-pollinating nature of pigeonpea, although some degree of outcrossing has been reported in it. Other self-pollinating species like tomato and wheat also show little polymorphism among accessions (Williams and Clair 1993; Joshi and Nguyen 1993). Further, it is likely that much of the morphological variation observed among germplasm is controlled by only a few genes. At present there is little genetic information available about diversity within pigeonpea cultivars. Previously, protein and isozyme electrophoresis were used for estimating 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. Our data demonstrates that the RAPD technique can be applied for estimating the genetic variability among closely related cultivars that is otherwise difficult to detect by other techniques.

Figure 3 depicts a dendrogram constructed from the similarity index values. Three different clusters can be observed, which are further divided into subgroups. The first cluster consists of 'Bahar' and 'DPPA 85-12', while the second cluster includes 'C-11', 'ICPL75-125' and 'T-21'. 'IPCL-75-125' and 'T-21' form a subgroup and are more closely related to each other than to 'C-11'. The third cluster shows two subgroups; the first consists of 'TAT-14' and 'PT-31', while the other subgroup consists of 'ICPL-87', 'MTH-12' and 'PT-14'. In the latter subgroup cvs 'MTH-12' and 'PT-14' are the most closely related on the basis of Nei's estimate of similarity, 0.91. Genetic variation at the DNA level is of prime importance in grouping genotypes to different heterotic groups, which can be of great relevance in assessing combining ability and developing maximum heterosis in pigeonpea. A high level of heterosis for yield has been observed in crosses between morphologically unrelated pigeonpea cultivars (Shrivastava et al. 1976). The estimate of genetic diversity can, therefore, be used in allocating the cultivars to different heterotic groups. Similar studies have been conducted in Brassica where the relationship between genetic diversity and heterosis has been examined using RAPD analysis (Jain et al. 1994).

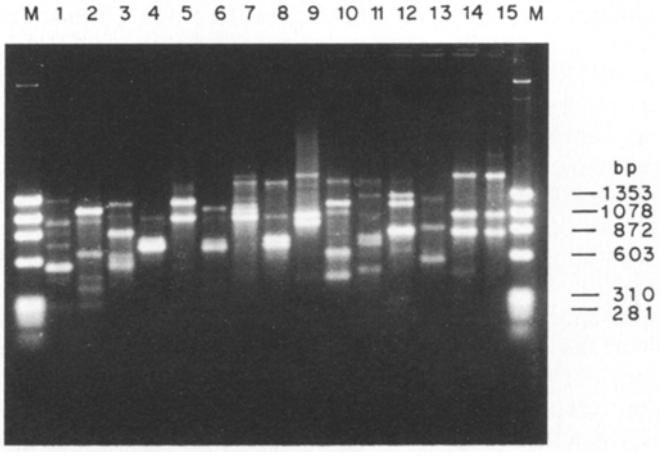
Fig. 3 Dendrogram constructed from the similarity index values for different pigeonpea cultivars



Genetic relationship among wild species

Several wild species representing the genus *Cajanus*, *Rhynchosia*, *Flemingia* and *Dunbaria* were selected to examine the potential of RAPDs for detecting interspecific variation. Unlike the situation found in the pigeonpea cultivars, extensive polymorphism was detected with all of the 16 primers in the wild genotypes. A total of 247 different RAPD products were considered for analysis. Many bands were common between the species, while numerous unique bands were also obtained. The total number of bands for the primers ranged from 8 (OPA4) to 25 (OPA16). Figures 4 and 5 show the electrophorogram of the PCR product amplified with primers OPA11 and OPA15, respectively. Table 2 indicates the number of polymorphic bands amplified by different primers. The similarity matrix between the wild species ranges from 0.22 to 0.85, which indicates that a large amount of genetic variation exists between the species. Species could clearly be dis-

Fig. 4 Agarose gel electrophoresis of DNA fragments amplified from different wild relatives of pigeonpea using primer OPA11. *Lane 1 C. acutifolius, 2 C. albicans, 3 C. goensis, 4 C. grandifolius, 5 C. lineatus, 6 C. reticulatus, 7 C. sericeus, 8 C. volubilis, 9 D. ferruginea, 10 R. rothii, 11 R. bracteata, 12 R. melacophylla, 13 F. stricta, 14 C. cajan-1, 15 C. cajan-2*



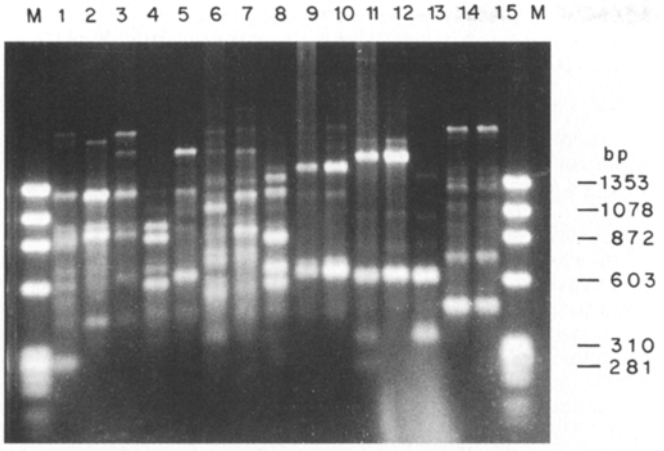


Fig. 5 Agarose gel electrophoretic pattern of DNA fragments amplified from different wild relatives of pigeonpea using primer OPA15. *M* represents the phi × 174 *Hae*III marker. *Numbers* at the *top of each lane* refer to the species listed in Fig. 4

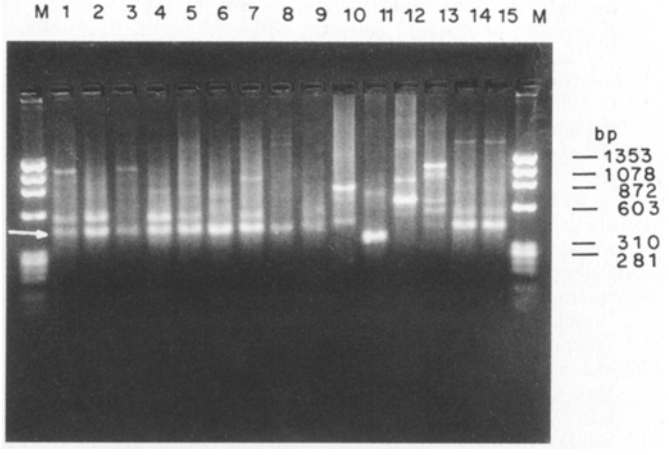
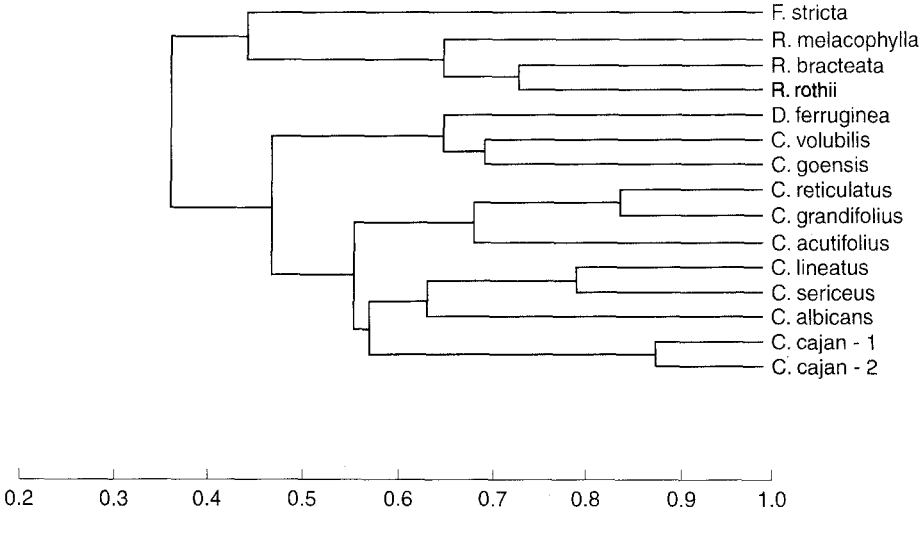


Fig. 6 Agarose gel electrophoretic pattern of RAPD fragments of wild species obtained with primer OPA-3. *Lanes 1–15* represent the wild species as mentioned in Fig. 4. A band, OPA3-500, common to all *Cajanus* species (*lanes 1–8*, *14* and *15*) is indicated by an *arrow*

criminated with as few as 1 well-selected primer or with 5–10 randomly chosen fragments. Interestingly, many highly informative markers were amplified. For example, primer no. 3 amplified a marker, OPA3-500, specific to all the species in the genus *Cajanus* but was absent in all the other genera (Fig. 6). Variation in the brightness of the bands was also observed within a gel. The possible causes include differences in template sequence copy number and varying degrees of mismatch between the primer and the binding site. The similarity matrix among the wild relatives was generated on the basis of shared RAPD amplification products, which includes information for all 247 bands, and was used for constructing a dendrogram (Fig. 7).

The subtribe *Cajaninae* consists of 13 genera with several species. The biosystematic relationships of these genera are not yet completely understood. All the phylogenetic studies in subtribe *Cajaninae* have been based on data obtained from morphological and cytological characters, breeding behaviour, seed storage protein pattern and RFLP data (Deodikar and Thakar 1956; Kumar et al. 1958; Ladizinsky and Hamel 1980; Reddy et al. 1980; Pundir and Singh 1985a,b; Nadimpalli et al. 1992). Within the genus *Cajanus* there are 32 species (Van der Masen 1986), the majority of which being from India and Australia. The transfer of new genes from other *Cajanus* species is well-advanced, with 11 wild species successfully crossed with *C. cajan*. Our data demonstrates the utility of RAPD in resolving biosystematic relationships in subtribe *Cajaninae*. The results from the dendrogram suggest that *C. albicans* clusters with *C. lineatus* and *C. sericeus*; with *C. lineatus* being more closely related to *C. sericeus* than to *C. albicans*. A previous report shows that all 3 species show crossability with *C. cajan* and a few characters have already been transferred from *C. sericeus* and *C. albicans* to *C. cajan* (Remanandan 1990). Our results indicate that the Australian species *C. acutifolius*, *C. reticulatus* and *C. grandifolius* are less closely related to *Cajanus cajan* and other Indian species. This is also supported

Fig. 7 Dendrogram constructed from the similarity index values for different wild species



from the view that the hybrids between pigeonpea and the Australian species tend to have a higher level of meiotic abnormalities (Dundas 1984). *C. reticulatus* and *C. grandifolius* are closer to each other than to *C. acutifolius*. *C. acutifolius*, which was previously known as *R. autifolia*, has now been merged in genus *Cajanus*. RAPD data also indicates the closer relationship of *C. acutifolius* with *Cajanus* species rather than with *Rhyncosia* species. *C. acutifolius* has been successfully crossed with *C. cajan*. In the present study, *C. goensis* grouped with *C. volubilis*, followed by *D. ferruginea*. All these species showed large amounts of variation as compared to *C. cajan*. *Rhynchosia* species (*R. rothii*, *R. bracteata* and *R. melacophylla*) grouped together, suggesting that all these species have been derived from a common ancestor. *Rhynchosia* species have several characters of agronomic importance, such as disease and insect resistance. For instance, *R. rothii* possesses the antimetabolic nature of protease inhibitor that can provide physiological resistance against certain insects (Singh and Jambunathan 1981). Some of the *Cajanus* species also possess very valuable characteristics that are lacking in pigeonpea cultivars: *C. scarabaeoides* has a resistance to the pod borer (*Heliothis armigera*), while *C. sericeus* and *C. albicans* are rich in protein (Reddy et al. 1980). Apart from these desirable traits, a unique quality like cytoplasmic male sterility, which is not easily available in the pigeonpea cultivars, has been transferred from the wild germplasm to the cultivars (Yadavendra 1986). Large amounts of interspecific variability and the ability of the species to cross-hybridize can make the task of reliably determining an individual's identity difficult at times. RAPD will be useful in determining the extent and role of introgression in the evolution of these species.

In summary, our study provides information on the molecular basis of the polymorphism detected by RAPD markers in pigeonpea. It demonstrates that markers generated via a RAPD assay can provide practical information for the management of genetic resource collection and identification.

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