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Phylogenetic relationships of Pigeonpea (*Cajanus cajan*) and its wild relatives based on RAPD markers

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In the present investigation RAPD marker was used for the elucidation of genetic relationships in the genus *Cajanus* and genetic fingerprinting of pigeonpea cultivars as well as wild species of *Cajanus*. RAPD markers utilized for the identification of pigeonpea, *Cajanus cajan* cultivars (DSLR-17, BDN-2, ICWR-03 and ICWR-12) and ten wild species, including *C. cajanifolius, C. lineatus, C. sericeus, C. acutifolius, C. lanceolatus, C. reticulates, C. albicans, C. scarabaeoides, C. volubilis* and *C. platycarpus*, using a set of 10 primers were found to be polymorphic at species level and generated 85 unequivocal scorable polymorphic bands. The size of amplification products ranges from 102 bp to 2854 bp. The present study accentuates upon the utility of RAPD markers for the identification of cultivars of pigeonpea and allied species of *C. cajan*. The inter/ intra specific genetic variability studies based on RAPD marker showed a large amount of genetic variation between the species of Cajanus and their clustering pattern partially, supported the sectional classification. It was hypothesised that both *C. cajan* and *C. cajanifolius* might be derived from a common ancestor and experienced minor genomic rearrangement during divergence.

Key words : *Cajanus*, Pigeonpea, RAPD, Proximity matrix analysis

INTRODUCTION

Pigeonpea, *Cajanus cajan* (L.) Millsp., is an important grain legume crop of the semi-arid tropics. *C.cajan* (L.) Millsp. is the only domesticated species under the subtribe Cajaninae Benth. of the tribe Phaseolae Benth. belonging to the subfamily Papilionoidae under the family Leguminosae (Bentham, 1965). After the inclusion of the Atylosia the genus Cajanus comprises 32 species, 18 of which are endemic to Asia, 13 to Australia, and one to West Africa (van der Maesen, 1986). Eleven related genera including Rhynchosia, Dunbaria and Flemingia have been described which can be considered to constitute the tertiary gene pool, while the *Cajanus* species showing crossability with the cultigen, constitute the secondary gene pool of the cultigen (van der Maesen, 1990). The genetic origin of pigeonpea is still not settled. Studies based on morphology (van der Maesen, 1980, 1986, 1990), cytology and crossability (Pundir and Singh, 1985b), isozymes (Krishna and Reddy, 1982) and nuclear RFLPs (Nadimpalli *et al.*, 1993) suggest a monophyletic origin from *C. cajanifolius*. On the other hand, the seeds storage protein profiles (Ladizinsky and Hamel, 1980; Jha and Ohri, 1996) and nuclear DNA amounts (Ohri *et al.*, 1994) suggest a polyphyletic origin of the cultigen. DNA based molecular markers have been used extensively to discern out the putative progenitor species and to depict phylogenetic relationships in several genera (Nadimpalli *et al.*, 1993; Ishii *et al.*, 1996). Randomly amplified polymorphic DNA (RAPD) is a dominant marker and it follows mendelian fashion. RAPDs are indefinite in number, capable of high level polymorphism and have been used in phylogenetic studies. RAPD has been extensively utilized in the study of genetic relatedness of plant cultivars and plant populations, as well as in the study of inter- and intra-specific genetic relationships between plant species. Within grain legume also crops RAPD markers have been widely used for the identification of genetic relationships among cultivars, among wild forms or between cultivars and wild forms. Ratnaparkhe *et al*.(1995) employed random amplified polymorphic DNA (RAPD) markers for the identification of *C. cajan* cultivars and the wild relatives of *C. cajan* and indicated the immense potential of RAPD marker in the genetic fingerprinting of pigeonpea cultivars and wild accessions. Present study reports here on the utilization of RAPD markers to elucidate the genetic relationships between *C.cajan* and its allied species.

MATERIALS AND METHODS

Plant materials:

Seeds of cultivars of pigeonpea (*Cajanus cajan* (L) Millsp.) BDN-2, DSLR-17, ICWR-03 and ICWR-12 and ten wild species (*C. cajanifolius, C. lineatus, C.*

sericeus, C. acutifolius, C. lanceolatus, C.reticulates, C. albicans, C. scarabaeoides, C. volubilis, C. platycarpus) were collected from ICRISAT, Patancheru, Andhra Pradesh. The species are maintained in the experimental garden of MITS, Rayagada, Orissa.

Extraction and quantification of genomic DNA :

Fresh and young leaf samples of equal quantity (~ 1.2g) were collected for isolation of genomic DNA. Genomic DNA was isolated and purified by using SDS method (Dellaporta *et al.*, 1983) with few modifications. DNA concentration and purity was measured by using UV–Vis spectrophotometer with TE buffer (pH 8.0) as blank. For further confirmation the quantification of DNA was accomplished by analyzing the purified DNA on 0.8% agarose gel along with diluted uncut lambda DNA as standard. DNA was diluted to concentration of 25ng/µl using TE buffer.

PCR Amplification using RAPD primers :

For RAPD analysis PCR amplification of 30 ng of genomic DNA was carried out using 10 standard decamer oligonucleotide primers (Operon Tech., USA). The Primers with their sequence information are given in Table 1 Each amplification reaction mix of 25µl contained the 30ng template DNA, 2.5µl of 10X assay buffer (100mM Tris.Cl, pH 8.3; 0.5 M KCl; 0.1% gelatin), 1.5 mM $MgCl_2$, 200µM each of the dNTPs, 20ng primers, 1.0 U Taq DNA polymerase (Bangalore Genei, India). The amplification was carried out in a thermal cycler with initial denaturation at 94°C for 5 min, followed by 45 cycles each consisting of denaturation at 94°C for 2 min, primer annealing at 37°C for 1 min and elongation at 72°C for 2 min. The final elongation was carried out at 72°C for 5 min with final hold at 10°C for infinite.

Electrophoretic and data analysis of Amplified products:

The PCR products were separated on 1.4% agarose gel containing Ethidium bromide solution (@ 0.5µg/ml of gel solutions) using TAE (40mM Tris acetate; 2mM EDTA) buffer at constant 50 V for about 4 hour. A gel loading buffer (20% Sucrose; 0.1 M EDTA, 1.0% SDS; 0.25% Bromophenol blue; 0.25% Xylene cyanol) was used as tracking dye. Amplified DNA fragments were visualized by UV transilluminator and photographed using photostation compact. The size of the amplicons were determined using Lambda DNA double digest, λ-EH, (Bangalore Genei) as standard and Total Lab software. Each amplified products were considered as unit character and the data were organized into 0-1 matrix and analyzed for proximity matrix using SPSS 8.0.1 software. The dendrogram or hierarchical cluster analyses were carried out using between group linkage method and squared elucidation distance interval. The information content of RAPD marker system was calculated for each marker and locus using the polymorphism information content (PIC), band informativeness (I_b) and resolving power (Rp) of the primer (Prevost and Wilkinson, 1999).

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below:

Generation of RAPD markers:

Amplification of all the 10 decamer primers (Table 1) used for RAPD analysis of four cultivars of *C. cajan*

Table 1: Polymorphism information and informativeness of RAPD primers used for analysis of nuclear genome diversity of *C. cajan* and its allied species

Sr. #	Primer	Primer sequence	No. of Loci amplified	No. of poly-morphic loci	%age poly-morphism	Amplicon size (bp)	PIC	Average band Informativeness (Av Ib)	Resolving power (Rp)
1.	OPA 01	5'-CAGGCCCTTC-3'	09	09		301-2854	0.975	0.285	2.564
2.	OPA 02	5'-TGCCGAGCTC-3'	06	06		448-1485	0.813	0.647	3.882
3.	OPA 03	5'-AGTCAGCCAC-3'	11	11		300-1584	0.919	0.454	5.002
4.	OPA 04	5'-AATCGGGCTG-3'	07	07		343-1702	0.833	0.658	4.606
5.	OPA 05	5'-AGGGGTCTTG-3'	08	08	100% in	288-1450	0.954	0.268	2.140
6.	OPA 06	5'-GGTCCCTGAC-3'	10	10	each	139-1490	0.937	0.372	3.722
7.	OPA 07	5'-GGTCCCTGAC-3'	12	12		512-1929	0.913	0.443	5.314
8.	OPA 08	5'-GTGACGTAGG-3'	11	11		102-1646	0.950	0.389	4.276
9.	OPA 09	5'-GGGTAACGCC-3'	08	08		480-1768	0.892	0.429	3.434
10.	OPA 10	5'-GTGATCGCAG-3'	03	03		278-641	0.948	0.144	0.432

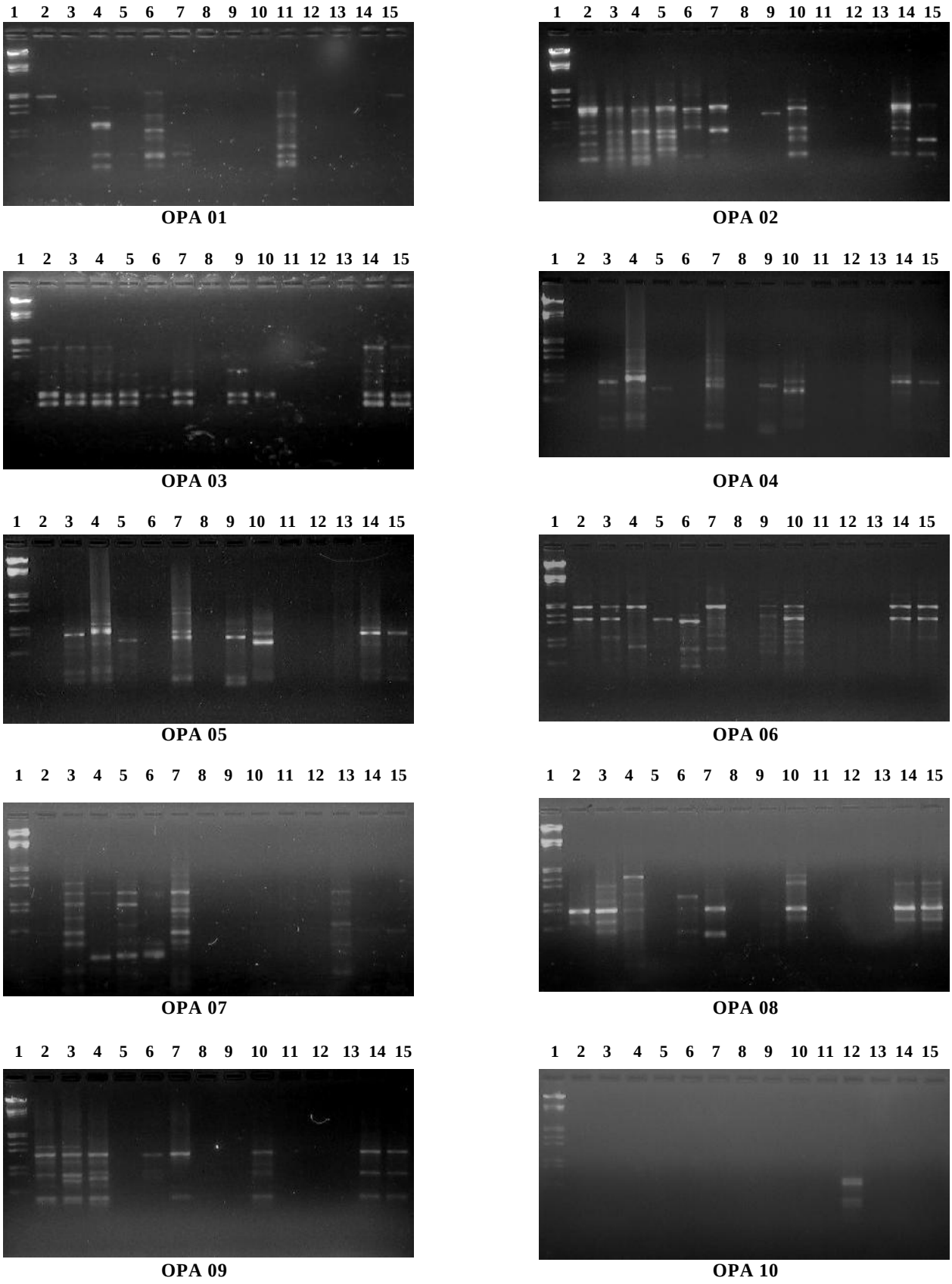

Fig. 1: Electrophoretic banding pattern of amplified products obtained with four different pigeonpea cultivars and 10 allied species of *Cajanus* using OPA primers

and ten different species of the genus *Cajanus* generated 85 unequivocal scorable polymorphic bands. The size of amplification products ranged from 102 bp to 2854 bp. Maximum 12 loci were amplified with primer OPA 07, whereas minimum three amplicons were observed with the primer OPA 10. No fragment was amplified in case of *C. volubilis* and *C. lineatus*. These 10 polymorphic primers exhibited variation with regard to average band informative ness (AvIb) and resolving power (Rp). Detailed RAPD banding pattern, resolving power of the primers, average band informative ness and polymorphic information content (PIC) are represented in Table 1. The primer OPA 04 showed highest AvIb (0.658) while OPA 10 showed lowest AvIb of 0.144. The primer OPA 07 showed highest Rp (5.314) and the primer OPA 10 showed lowest Rp (0.432) values. All the 10 primers exhibited high PIC values. But among them, OPA 01 showed high PIC (0.975) and OPA 02 showed low PIC (0.813) values. In the present study no single primer was able to distinguish between all the four cultivars and ten wild species. However, amplification by different primers was informative for the identification of three cultivars as well as seven allied species (Table 2). The markers OPA01-1833, OPA01-1081, OPA02-641, OPA02-554, OPA02-278, OPA03-1584, OPA03-1183, OPA03-701, OPA03-301, OPA04956, OPA04-856, OPA04-703, OPA05-1450, OPA05-1244, OPA05-1021, OPA05-689, OPA06-1490, OPA06-1338, OPAO06-1046, OPA06-713, OPA06-139, OPA07-1420, OPA07-1314, OPA07-1138, OPA 07-512, OPA08-1646, OPA08-862, OPA09-1160, OPA09-1108 and OPA 09-480 were unique to different species of Cajanus while, OPA05-914, OPA05-288, OPA06-926, OPA06-330, OPA08-1450, OPA08-585, OPA10-725 were unique to the cultigens used in the present study.

Genetic relationship within Cajanus cajan:

The proximity matrix indices was estimated among the four cultivated accessions of *C. cajan* to quantify the level of polymorphism for intraspecific studies. The proximity matrix indices ranges from 0.717 to 1.0 (Table 3), indicating less genetic variation between cultivars. Among the cultivars, ICWR 3 and ICWR12 are pretty close to each other while, DSLR17 and ICWR3 are distantly related to each other. Genetic variation at the DNA level is of prime importance in grouping genotypes into different heterotic groups, which can be of great relevance in assessing combining ability and developing maximum heterosis in pigeonpea. A dendrogram constructed from the proximity matrix indices values (Fig. 2). One single cluster was formed with ICWR 3, ICWR 12 and BDN 2, and DSLR 17 was out grouped. ICWR3 and ICWR 12 form a subgroup in the cluster and are more closely related to each other than to BDN 2.

Table 2: Primer response for the identification of *C. cajan* cultivars and the allied species of *Cajanus*

Sr. #	Species/Cultivar	Primer	No. of amplicons	Marker (s)
1.	*C. cajan* DSLR 17	OPA 02, OPA 05, OPA 08	03	OPA02-725, OPA05-288, OPA08-1450
2.	*C. cajan* ICWR3	OPA 05, OPA 06	03	OPA05-914, OPA06-926, OPA06-330
3.	*C. cajan* ICWR12	OPA 08	01	OPA08-1450
4.	*C. cajanifolius*	OPA 01, OPA 04, OPA 05, OPA 06, OPA 09	06	OPA 01-1833, OPA01-1081, OPA04-856, OPA05-1021, OPA06-1046, OPA09-1160,
5.	*C. scarabaeoides*	OPA 03, OPA 04	03	OPA03-701, OPA03-301, OPA04-956
6.	*C. platycarpus*	OPA 04, OPA 07, OPA09	04	OPA04-703, OPA07-1420, OPA 07-512, OPA09-1108
7.	*C. albicans*	OPA 05, OPA 06, OPA08, OPA 09	07	OPA05-1450, OPA05-1244, OPA06-1490, OPA06-1338, OPA08-1646, OPA08-862, OPA09-480
8.	*C. sericeus*	OPA 03, OPA 06	02	OPA03-1183, OPA06-139
9.	*C. acutifolius*	OPA 03, OPA 05, OPA06, OPA 07	05	OPA03-1584, OPA05-689, OPA06-713, OPA07-1314, OPA07-1138
10.	*C. lanceolatus*	OPA 10	03	OPA10-641, OPA10-554, OPA10-278

Table 3 : Proximity matrix index based on 1-0 binary matrix of RAPD marker data generated for four pigeonpea cultivars

Case	C. cajan BDN-2	C. cajan DSLR-17	C. cajan ICWR03	C. cajan ICWR12
C. cajan BDN-2	1.000			
C. cajan DSLR-17	0.777	1.000		
C.cajan ICWR03	0.861	0.717	1.000	
C.cajanI CWR12	0.823	0.907	1.000	1.000

Genetic relationship in the genus Cajanus:

The proximity matrix indices were estimated among the species of Cajanus using 85 RAPD markers to quantify the level of polymorphism for inter-specific studies. The pair wise proximity matrix indices values ranged from 0.002 to 0.574 (Table 4), which indicates large amount of genetic variation exist between the species of *Cajanus* at the DNA level. Dendrogram constructed from proximity matrix data exhibited the clustering of *C. cajan* accessions with *C. cajanifolius* (Section- *Cajanus*) in one cluster, while the wild *Cajanus* species except *C. acutifolius* belonging to the secondary and tertiary gene pool form another cluster, respectively (Fig. 3). *C.platycarpus* (sec. Rhynchosoides) is found to be out grouped from its major cluster justifying its status in the tertiary gene pool. RAPD data indicates *C. reticulatus* and *C. lanceolatus* are close to each other than to *C. acutifolius* and *C.acutifolius* showed close relationship with *C. cajan* genotypes and *C. cajanifolius*. The results from the dendrogram indicates that species belonging to Atylia (*C. lineatus* and *C. sericeus*), Cantharospermum (*C. albicans* and *C. scarabaeoides*) and Fruticosa (*C. acutifolius*, *C. lanceolatus* and *C. reticulates*) not formed any close subclusters. All these species showed a large amount of genetic variation as compared to *C. cajan* and their clustering pattern partially, supported the sectional classification suggested by van der Maesen (1986). Again from the studies it has presumed that both *C.cajan* and *C.cajanifolius* might be derived from a common ancestor and experienced minor genomic rearrangement during the course of evolution.

Ratnaparkhe *et al.* (1995) also detected several RAPD markers for the identification of pigeonpea cultivars as well as the allied species of *Cajanus*. However, the primer set, cultivar set and allied species were different in both the studies. No other information are available on the identification of pigeonpea cultivars and wild species at DNA level. As a result, pigeonpea breeding relies heavily on a phenotypic selection method. Secondly, pigeonpea is one of the exception among grain

Table 4: Proximity matrix index based on 1-0 binary matrix of RAPD marker data generated for four *C. cajan* cultivars and 10 wild species in the genus *Cajanus*

Case	C. cajan BDN-2	C. cajan DSLR-17	C. cajanifolius	C. scarabaeoides	C. platycarpus	C. albicans	C. volubilis	C. sericeus	C. acutifolius	C. lineatus	C. lanceolatus	C. reticulatus	C. cajan ICWR3	C. cajan ICWR12
C. cajanBDN-2	1.000													
C. cajanDSLR-17	0.777	1.000												
C. cajanifolius	0.501	0.516	1.000											
C. scarabaeoides	0.333	0.396	0.238	1.000										
C. platycarpus	0.362	0.171	0.282	0.237	1.000									
C. albicans	0.420	0.606	0.358	0.511	0.203	1.000								
C. volubilis	0.213	0.213	0.213	0.213	0.213	0.213	1.000							
C. sericeus	0.295	0.309	0.297	0.378	0.110	0.436	0.213	1.000						
C. acutifolius	0.515	0.473	0.332	0.118	0.150	0.315	0.213	0.403	1.000					
C. lineatus	0.057	0.000	0.227	0.083	0.574	0.220	0.213	0.094	0.037	1.000				
C. lanceolatus	0.094	0.050	0.046	0.113	0.090	0.042	0.213	0.122	0.078	0.159	1.000			
C. reticulates	0.026	0.558	0.051	0.562	0.132	0.439	0.213	0.070	0.002	0.129	0.149	1.000		
C.cajan ICWR3	0.861	0.717	0.750	0.195	0.218	0.556	0.213	0.403	0.575	0.037	0.078	0.002	1.000	
C.cajanICWR12	0.823	0.907	0.792	0.252	0.217	0.548	0.213	0.381	0.437	0.057	0.094	0.140	1.000	1.000

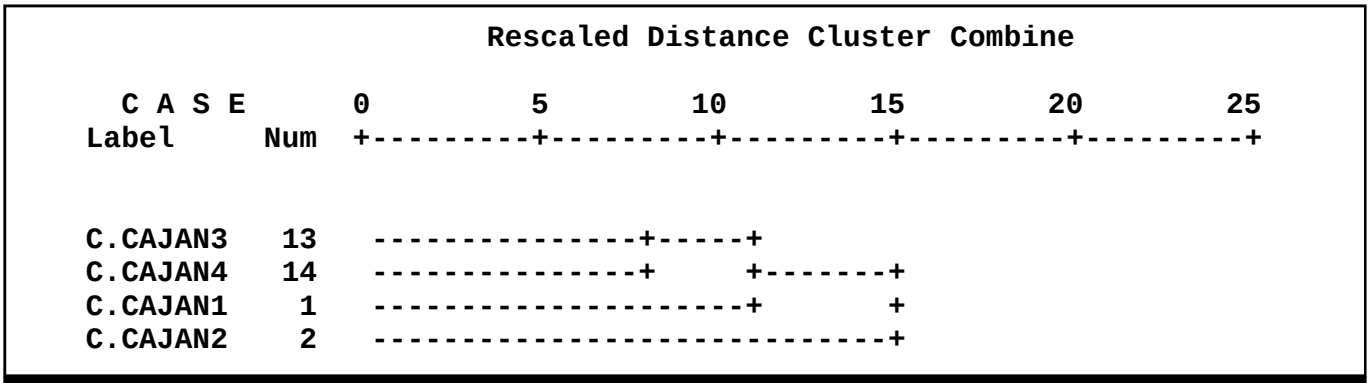

Fig. 2: Dendrogram of four cultivars of pigeonpea based on proximity matrix indices of RAPD marker data

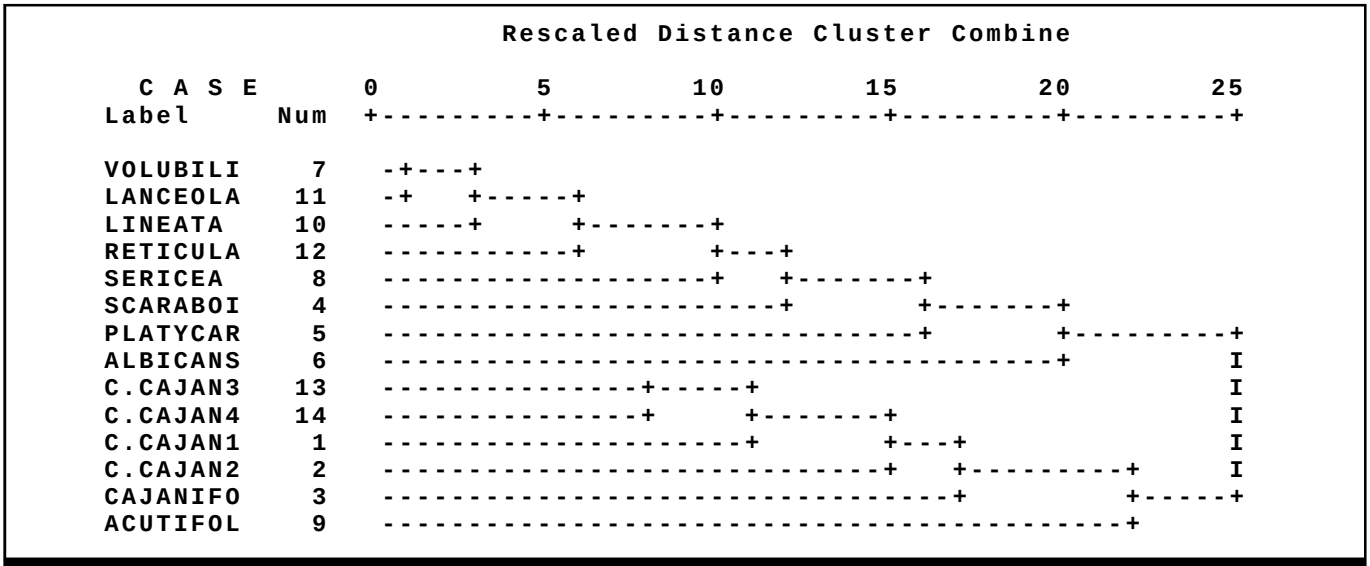

Fig. 2: Dendrogram of four cultivars of pigeonpea and 10 allied species in the genus *Cajanus* based on proximity matrix indices of RAPD marker data

legumes in that it has tendency towards frequent out crossing due to which existing standard cultivars have become heterogeneous for several important agronomic characters such as disease resistance. The identification of cultivars will also be helpful in assessing the purity and stability of the genotypes entering into the breeding programme. Similarly, the species could clearly, be distinguished with as few as one selected primer or with 0-7 polymorphic amplicons. These species specific markers may also be utilized to track the introgressive wide hybridization programme for the genetic augmentation in pigeonpea. In the present investigation the RAPD marker were used not only for the elucidation of genetic relationship in the genus Cajanus but also for the genetic fingerprinting of pigeonpea cultivars as well as wild species of *Cajanus*. In addition, from the present study it has also been demonstrated that markers generated via RAPD assay can provide practical information for the management of germplasm collections and precise identification cultivars as well as its allied species.

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