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Genetic Diversity of Pigeonpea (*Cajanus cajan* (L.) Millsp.) Cultivars and Its Wild Relatives Using Randomly Amplified Polymorphic DNA (RAPD) Markers

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ABSTRACT

Genetic diversity among and between 16 cultivars of pigeonpea (*Cajanus cajan* (L) Millsp.) and its wild relatives (*C. albicans* and *C. lineatus*) analysed using RAPD. Twenty two random primers with an average of 71.2% polymorphism produced 151 polymorphic bands. Cluster analysis based on these 151 RAPD markers revealed relatively low level (0.434 - 0.714) of genetic diversity among cultivars and high level of diversity between cultivars and wild relatives. *C. albicans* and *C. lineatus* showed only 0.231 similarity with each other and *C. albicans* showed relatively higher similarity with *C. cajan* cultivars than that showed by *C. lineatus*. In dendrogram the 16 cultivars grouped into two distinct clusters comprising of seven and nine genotypes each while the wild species form out groups. Bootstrap analysis of the dendrogram was performed and resulted in significant bootstrap values. Principal components analysis (PCA) also revealed the similar results that of unweighted pair group method with arithmetic mean (UPGMA). The first, second and third PCs contributed 55.9%, 5.9%, and 5.6% of the variation, respectively, with cumulative variation of the first three PCs was 67.4%.

Keywords: Genetic Diversity; RAPD; Pigeonpea

1. Introduction

Pulses are leguminous crops harvested exclusively for dry grains. Genetic improvement in pulses production could not achieved significantly in comparison to other cereal crops through conventional breeding methods. Major yield constraints are high susceptibility of pulses to biotic and abiotic stresses, and high genotype × environment interaction on the expression of important quantitative traits which lead to slow gain in genetic improvement and yield stability of pulses [1]. Marker assisted conventional breeding may be the alternative for increasing productivity. For this the available high yielding diverse lines should be used as base material for incorporating some traits from unadapted cultivars, lines or wild relatives [2]. A starting point therefore, is to determine the extent of genetic diversity within these available lines for the selection of parental lines and design breeding strategies [3].

Pigeonpea (*Cajanus cajan* (L.) Millspaugh) is tall, woody, perennial legume with centre of diversity in India [4]. It is only cultivated food crop of the cajaninae sub-tribe and popularly known as redgram (Arhar or Tur). It has diploid genome (2n = 22) and estimated at about 0.853 pg [5]. Pigeonpea is an important pulse crop of India but as compared to other grain legumes it has received relatively little research attention.

Earlier morphological markers have been used for assessment of genetic diversity using cultivated pigeonpea and wild relatives [6]. With the development of environmentally neutral, reliable and plant growth independent molecular markers, many researchers initiated the pigeonpea genetic diversity analysis. Assessment of genetic variability has been done using various molecular markers viz., RAPD [7-9], amplified fragment length polymorphism (AFLP) [3,10,11], restriction fragment length polymorphism (RFLP) [11,12], simple sequence repeat (SSR) [11,13], PCR-RFLP [14]. The present study was therefore, aimed to assess genetic diversity among pigeonpea cultivars and its related wild species using RAPD markers.

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2. Materials and Methods

2.1. Plant Material

Sixteen cultivars of *C. cajan* and two wild relatives (*C. albicans* and *C. lineatus*) were procured from core collection maintained at Indian Institute of Pulses Research, Kanpur, India (**Table 1**).

2.2. Genomic DNA Extraction

Pigeonpea seeds were surface sterilized with 0.2% $HgCl_2$ and washed 3 - 4 times and grown in pots in sunlight for three weeks. DNA was isolated from young leaves using HiPurA™ Plant genomic DNA Miniprep Purification spin kit (Himedia Laroratories Pvt. Ltd). Quality and quantity of isolated DNA was checked by spectrophotometry as well as by 0.8% agarose gel electrophoresis. The DNA yield obtained was in the range of 1.0 µg to 3.0 µg.

2.3. PCR-RAPD Analysis

The PCR was performed in a reaction volume of 25 µl containing 10 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 200 µM of each dNTP, 4 µM of primer, 1 Units of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India) and 30 ng of genomic DNA. A total of 30 decamer primers (GC content 60% - 90%) were used for RAPD analysis. Out of 30 primers 19 belong to Operon series (Operon Technologies USA) and 11 were selected from the literature (**Table 2**). The PCR amplification was carried out for 45 cycles in a thermal cycler (PTC-200, Bio-Rad USA). The reaction had initial denaturation step at 94˚C for 5 min, followed by 45 cycles of 94˚C for 1 min, annealing temperature (Tm-5˚C) for 1 min, 72˚C for 2 min. The final extension step was at 72˚C for 5 min. Amplified products were separated on 1.5% agarose gels having 0.5 $\mu g \cdot ml^{-1}$ of the ethidium bromide at 50 V for 3 h. The gels were observed under a UV light source in a gel documentation system (BIOVIS Gel, Expert Vision Labs Pvt. Ltd, India).

2.4. Data Analysis

Band positions in comparative RAPD profiles for each

Table 1. List of genotypes and the wild relatives of pigeonpea (*Cajanus cajan* (L.) Millsp.) used for genetic diversity analysis.

S. No.	Genotype	Pedigree	Maturity duration (days)	Special features
1.	ICPL-87 (Pragati)	T21 × JA277	116 - 126	Determinate, brown seeded, spreading, resistant to wilt
2.	AK-101	Selection from germplasm line	145 - 160	Indeterminate, semi-spreading, tolerant to wilt.
3.	Vamban-1	(Prabhat × HY3A) × (T-21 × 102)	95 - 100	Determinate, suitable for peanut intercropping
4.	Jawahar (JKM-7)	ICP8863 × LRG30	173 - 180	Tolerant to wilt and Phytophthora blight
5.	ICPL-85063 (Laxmi)	BDN-1 × (T-21 × JA275)	160 - 200	Semi-spreading, suitable for rabi planting also
6.	Azad	Bahar × NP (WR) 15	153 - 210	Indeterminate, resistant to sterility mosaic semi-spreading
7.	Pusa-2002	P945 × Pusa-78	130 - 150	Early maturing, indeterminate
8.	BDN-2	Sel. from local bori11-132-A-1	150 - 160	Indeterminate, tolerant to wilt
9.	GT-101	BWR-24 × Pusa Sweta	133 - 185	Indeterminate, semi-spreading
10.	Malviya-Vikalp (MA-3)	Sel. from land races no. MA-2	178 - 262	Spreading, constricted pod, resistant to pod fly
11.	C-11	Sel. from sanga Reddy (A.P.)	195 - 200	Profuse branching, brown seeded, tolerant to wilt
12.	Manak	T-21 × UPAS120	120 - 130	Indeterminate, semi-spreading, small seeded
13.	Paras (H82-1)	EE76 × UPAS120	133 - 145	Indeterminate, tolerant to wilt
14.	Birsa Arhar	Local sel. Land races Ranchi	180 - 200	Resistant to wilt under field condition
15.	Pusa- 84	Pusa Ageti × T-21	140 - 150	Semi-spreading, determinate, semi tall, brown seeded
16.	T-15-15	Sel. From land races	200 - 210	Indeterminate, white seeded, suitable for vegetable purpose
17.	*C. albicans* (wild)	NKR 185	-	-
18.	*C. lineatus* (wild)	JM 3366	-	-

Table 2. Random primers selected for RAPD analysis.

S. No.	Primer Name	Sequence (5'-3')	GC %	Total number of bands amplified	Number of polymorphic bands	% polymorphism
1.	OPA-09	GGGTAACGCC	60	*	*	*
2.	OPB-14	TCCGCTCTGG	70	8	5	62.50
3.	OPB-17	AGGGAACGAG	60	10	9	90.00
4.	OPB-19	ACCCCCGAAG	70	8	7	87.50
5.	OPC-05	GATGACCGCC	70	17	11	64.70
6.	OPH 02	TCGGCACGCA	70	7	5	71.00
7.	OPH-03	AGACGTCCAC	60	*	*	*
8.	OPH-05	AGTCGTCCCC	70	*	*	*
9.	OPH 10	CCTACGTCAG	60	*	*	*
10.	OPH11	CTTCCGCAGT	60	*	*	*
11.	OPH-12	ACGCGCATGT	60	8	4	50.00
12.	OPM-07	CCGTGACTCA	60	10	8	80.00
13.	OPP-07	GTCCATGCCA	60	8	5	62.50
14.	OPP 09	GTGGTCCGCA	70	10	6	60.00
15.	OPAQ-05	ACGGAGCTGA	60	*	*	*
16.	OPAQ-18	GGGAGCGAGT	70	7	1	14.00
17.	OPAQ-19	AGTAGGGCCT	60	*	*	*
18.	OPAZ-05	TCCGCATACC	60	2	2	100.00
19.	OPAZ-18	CCGACGTTGA	60	4	3	75.00
20.	P-23	GTAGGCGTCG	70	15	10	66.60
21.	P-24	GGCTCGTACC	70	13	12	92.30
22.	P-25	GACCCCGGCA	80	12	7	58.33
23.	P-26	CAGGGGACGA	70	10	9	90.00
24.	P-27	CGCCACGTTC	70	13	10	76.92
25.	P-28	GCCTCCTACC	70	12	8	66.92
26.	P-29	GGCGTCGGGG	90	8	7	87.50
27.	P-30	CAGGGCCGCT	80	9	7	77.77
28.	P-31	CTCTCCGCCA	70	10	7	70.00
29.	P-32	CTCGGCTGGA	70	*	*	*
30.	P-33	AGGCCCGATG	70	11	8	72.00

Primers starting with the letter OP are operon primers. Primers starting with P are non-operon primers. *Primers did not produce reproducible bands.

genotype and primer combination were scored. RAPD profiles from only those genotype x primer combinations, which generated consistent bands after amplification, were included in this study. A band was equated to a marker; a score of "1" was given for its presence, and "0" was assigned for absence. Jaccard's similarity coefficient [15] was estimated from these binary data using FreeTree [16] software. The resulting similarity matrix was used for UPGMA based dendrogram construction. Support for clusters obtained was evaluated by bootstrap analysis

with FreeTree and Tree View [17] software through generating two thousand samples by re-sampling. PCA was also done to check the results of UPGMA based clustering using EIGEN module of NTSYSpc [18].

3. Results and Discussion

3.1. Polymorphism and Marker Efficiency

Out of the 30 primers tested, 22 produced reproducible and polymorphic patterns (**Table 2**). The 22 primers yielded a total of 212 fragments, of which 151 amplicons (71.2%) were polymorphic, the number of polymorphic bands per primer ranged from 2 to 17, the average being 9.63 (**Table 3**). Primers OPB17, OPB-19 OPM-07, OPAZ-05, OPAZ-18, P-24, P-26, P-27, P-29, and P-30 were the most informative primers as 75% or more of the amplicons were polymorphic. Gel image of RAPD profile of primer P-27 is given in **Figure 1**. These ten highly polymorphic primers produced 83.1% polymorphism (**Table 3**). Present study obtained 71.2% polymorphic band with an average 9.63 band per primer which is comparable to earlier studies on pigeonpea using RAPD marker [8,9].

3.2. Genetic Diversity

Genetic similarities measured through analysis of data on the 151 RAPD markers revealed varying degrees of genetic relatedness among wild and cultivated genotypes. Jaccard's similarity coefficients between species *i.e.*, *C. lineatus* and *C. cajan* cultivars ranged from 0.148 to 0.220 and between *C. albicans* and *C. cajan* ranged between 0.270 and 0.377 (**Table 4**). The genetic similarity between *C. lineatus* and *C. albicans* was 0.231. Jaccard's similarity coefficients among *C. cajan* cultivars ranged from 0.434 - 0.714 (**Table 4**). Lowest similarity was obtained between GT-101 and Azad (0.434) followed by that between Birsa arhar and Vamban-1 (0.451). Highest similarity was obtained between Pusa-2002 and Manak (0.714) followed by that between ICPL-87 and Azad (0.703).

The genetic diversity range obtained in this study is higher (0.434 - 0.714) comparable to the previous reports on pigeonpea based on RAPD [9] and AFLP [10] markers. Some researchers [13,19] developed characterized

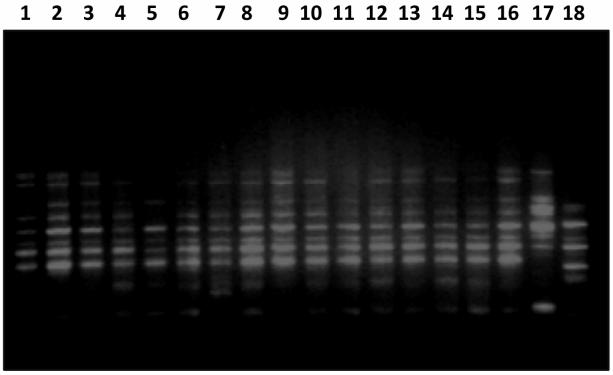

Figure 1. Gel image showing RAPD profile obtained by P-27 primer. Lane 1-18 represents the genotypes (Genotypes name is given in Table 1).

Table 3. Summary of amplification patterns generated by the random primers tested in this study.

Description	Number/frequency
Total number of primers screened with all the eighteen pigeonpea genotypes	30
Number of primers that produced polymorphic bands	22
Total number of bands amplified by the primers that generated polymorphic bands	212
Average number of bands per primer	9.63
Total number of polymorphic bands	151
Percentage of polymorphic bands	71.22%
Average number of polymorphic bands per primer	6.8
Total number of primers that produced more than 75% polymorphic bands	10
Total number of bands produced by these 10 primers	95
Number of polymorphic bands produced these 10 primers	79
Percentage of polymorphic bands	83.15%
Average number of polymorphic bands per primer	7.9
Average size of the fragments amplified	4000 bp - 300 bp

Table 4. Jaccard's similarity coefficient of the 16 pigeonpea cultivars and two related wild species based on 151 RAPD markers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	1.000																	
2	0.611	1.000																
3	0.494	0.662	1.000															
4	0.632	0.523	0.487	1.000														
5	0.536	0.647	0.616	0.482	1.000													
6	0.703	0.539	0.505	0.680	0.516	1.000												
7	0.633	0.520	0.458	0.604	0.580	0.579	1.000											
8	0.569	0.593	0.579	0.592	0.586	0.623	0.651	1.000										
9	0.530	0.674	0.625	0.477	0.612	0.434	0.556	0.612	1.000									
10	0.647	0.600	0.568	0.620	0.559	0.630	0.569	0.686	0.585	1.000								
11	0.604	0.629	0.561	0.632	0.659	0.604	0.651	0.622	0.546	0.666	1.000							
12	0.554	0.560	0.462	0.536	0.571	0.551	0.714	0.537	0.563	0.543	0.571	1.000						
13	0.702	0.690	0.581	0.536	0.625	0.569	0.617	0.606	0.689	0.560	0.625	0.609	1.000					
14	0.670	0.549	0.451	0.602	0.526	0.675	0.682	0.595	0.505	0.620	0.613	0.654	0.654	1.000				
15	0.612	0.568	0.474	0.511	0.530	0.560	0.606	0.612	0.571	0.655	0.612	0.580	0.633	0.678	1.000			
16	0.526	0.620	0.551	0.470	0.526	0.488	0.537	0.560	0.659	0.500	0.543	0.579	0.695	0.623	0.678	1.000		
17	0.299	0.352	0.333	0.270	0.311	0.284	0.308	0.349	0.349	0.277	0.275	0.320	0.320	0.310	0.336	0.377	1.000	
18	0.217	0.148	0.166	0.182	0.166	0.168	0.184	0.147	0.170	0.188	0.176	0.181	0.161	0.203	0.220	0.192	0.231	1.000

Name of genotypes 1 to 18 is given in **Table 1**.

and utilized SSR marker and obtained very low range of alleles per locus as compared to other legume crops such as soybean (11 - 26 alleles per locus) [20]. We have recently obtained greater range of genetic diversity (0.29 - 0.88) among pea genotypes [21] as compared to our present work in pigeonpea using RAPD markers. After considering all the results and previous reports we can conclude that pigeonpea genotypes possesses very narrow genetic diversity as compared to wild relatives and RAPD markers seem to be an efficient marker system in pigeonpea as compared to other markers.

In the dendrogram 16 genotypes were grouped into two distinct clusters; cluster I and cluster II with seven and nine genotypes, respectively, whereas the two wild species did not group into clusters (**Figure 2**). In the dendrogram, Cluster I consisted of GT-101, Paras, T-15-15, AK-101, Vamban-1, C-11, ICPL-85063. Nine genotypes of cluster II were JKM-7, Azad, ICPL-87, BDN-2, MA-3, Birsa arhar, Pusa-84, Manak, and Pusa-2002. Some of the genotypes of cluster II *viz.*, ICPL-87, JKM-7, BDN-2, and Birsa arhar are resistant to wilt and JKN-7, BDN-2, Birsa arhar along with MA-3 belonged to similar agro-climatic region (M.P., Gujarat, Maharashtra); this may be the reason for showing more similarity with each other and clustering together. Bootstrap analysis was used to evaluate the degree of support for clusters within dendrogram. The two major branching points that differentiated the *C. lineatus* and *C. albicans* had bootstrap values 100 and 92, respectively. The branch point that grouped the all *C. cajan* cultivars into two clusters has bootstrap value of 100. These values show that the dendrogram obtained from RAPD markers is robust and differentiation of genotypes is precise and accurate. Only four branches showed bootstrap value less than ten. PCA revealed that the PC1, PC2, and PC3 accounted for 55.9%, 5.9%, and 5.6% of the variation, respectively. Together, the first three PCs accounted for 67.4% of the total variation. Two dimensional (**Figure 3**) and three dimensional (**Figure 4**) plots were prepared by using the first two and first three PCs, respectively. In 2-D plot the wild species *C. lineatus* and *C. albicans* occupied distant positions from the *C. cajan* cultivars which in turn showed more similarity with each other and grouped together. The 3-D plot differentiated the all 16 cultivars into three clusters while wild species placed distant position. Cluster I comprised the genotypes Vamban-1, GT-101, AK-101,

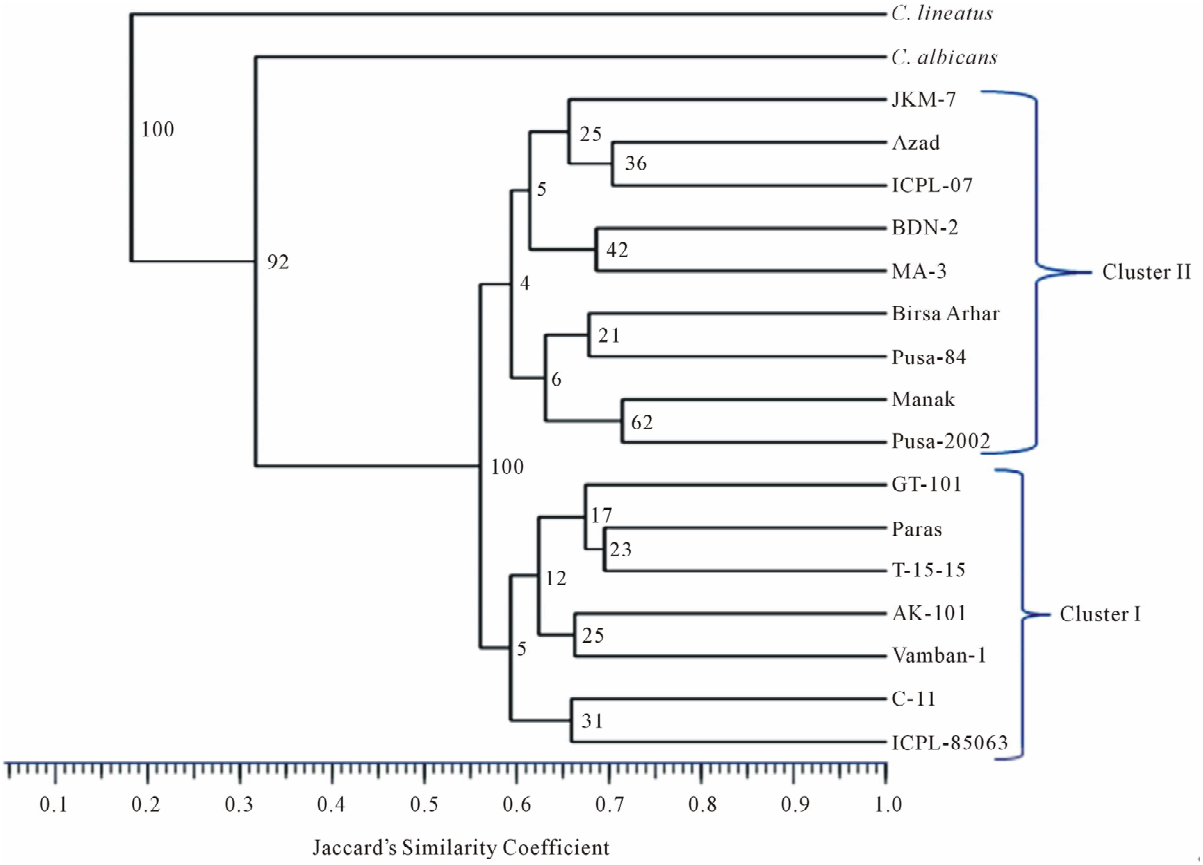

Figure 2. Dendrogram of pigeonpea along with wild relative showing genetic similarity.

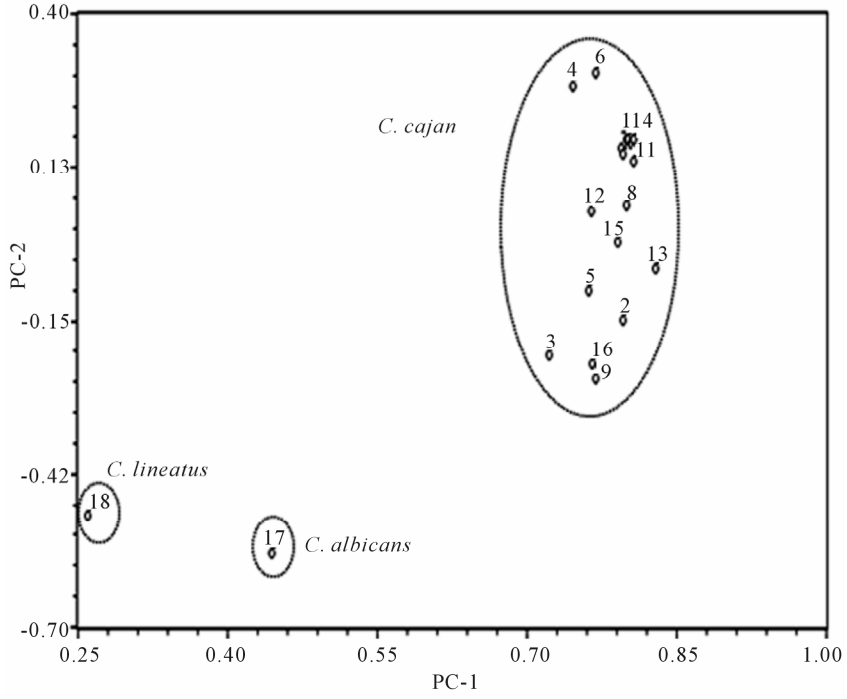

Figure 3. Two dimensional plot of principal components 1 and 2 based on RAPD markers of pigeonpea genotypes. Name of the genotypes 1 to 18 is given in Table 1.

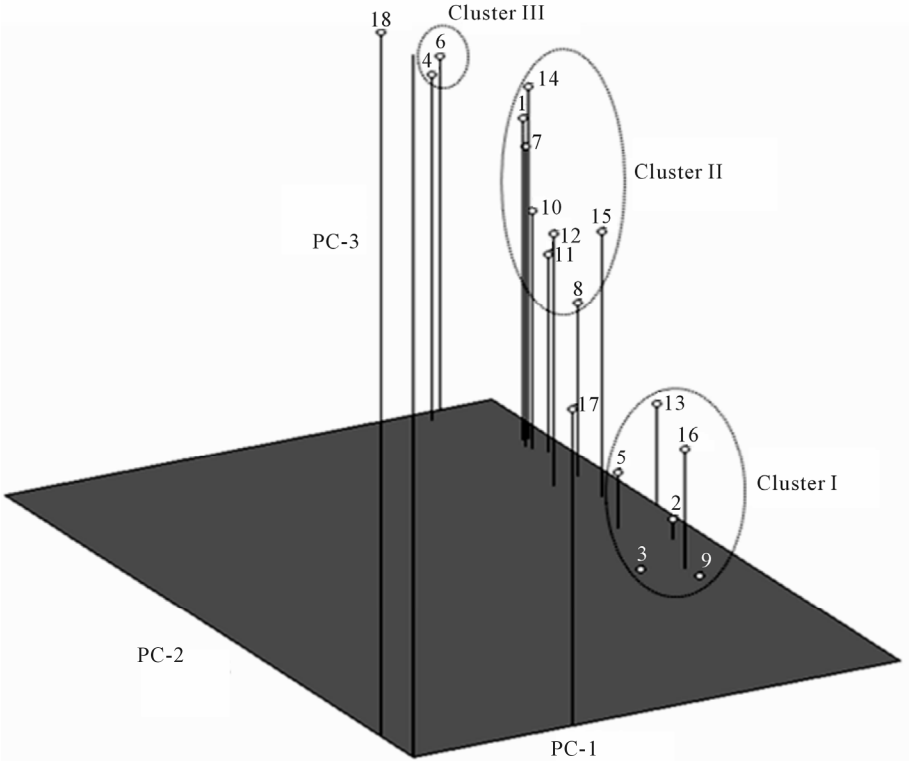

Figure 4. Three dimensional plot of principal components 1, 2 and 3 based on RAPD markers of pigeonpea genotypes. Name of the genotypes 1 to 18 is given in Table 1.

ICPL-85063, T-15-15 and Paras. Cluster II included eight genotypes viz., BDN-2, Pusa-84, C-11, Manak, MA-3, Pusa-2002, ICPL-87 and Birsa arhar. The cluster III included genotypes JKM-7 and Azad. The result of PCA was similar to that obtained by UPGMA clustering. All the genotypes (except C-11) of cluster I of PCA 3-D plot grouped in cluster I of UPGMA dendrogram. Similarly cluster II of PCA 3-D plot and cluster II of the UPGMA dendrogram shared most of genotypes except JKM-7 and Azad. JKM-7 and Azad are present in cluster II of UPGMA but in PCA 3-D plot these genotypes comprised a distinct cluster III. Genotype C-11 showed different grouping pattern in both the methods. In UPGMA it grouped in cluster I while in PCA 3-D plot it clustered with genotypes of cluster II. Thus both the hierarchical method and the ordination method resulted in similar type of clustering pattern which is similar to earlier studies on pea [21,22] therefore it can be suggested that both UPGMA and PCA should be performed for genetic diversity analysis. Present study also resulted in some pigeonpea specific marker. Primers OPP-09, P-23, P-24, P-25, P-27, P-28, P-29, P-30 amplified nine intense, distinct, species-specific markers. These markers were present in all the 16 genotypes but absent in both the wild species. One such marker that was amplified by primer P-25 is shown in **Figure 5**. These markers can be utilized for development of more specific SCARs in pigeonpea.

In the present study we obtained higher diversity between the wild species and between wild and cultivated genotypes. One report [23] utilizing diversity arrays technology (DArT) obtained similar results with 96 accessions representing nearly 20 species of *Cajanus* including the cultivated one. It was concluded that most of

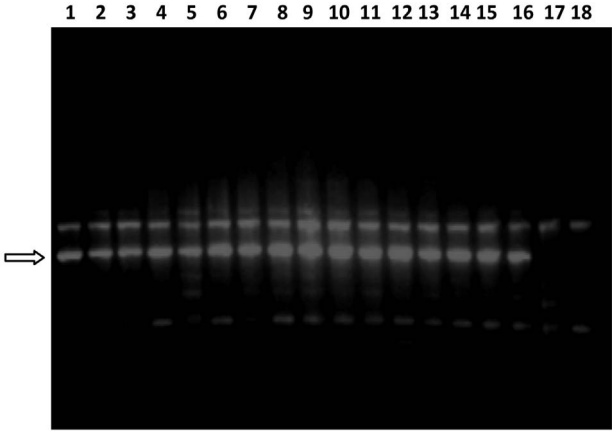

Figure 5. Gel image showing RAPD profile obtained by P-25 primer Lane 1 - 18 represents the genotypes (Genotype name is given in Table 1). Arrow showing the position of species specific marker which is present in all the 16 pigeonpea cultivars but absent in both the wild relatives.

the diversity was among the wild relatives of pigeon pea or between the wild and the cultivated species, but not among the cultivated accessions. Very low level of genetic diversity in pigeonpea cultivras was reported also by many other researchers [9,10,13]. Such narrow genetic base can put a serious impediment to breeding progress in pigeon pea [23].

The reason for narrow genetic diversity in pigeonpea and in other pulses could be that only few genotypes with high degree of relatedness have been used as parents in crossing programmes for the development of new cultivars which leads to narrowing down the genetic base of cultivated germplasm of pulses [24]. One possible reason behind narrow genetic diversity in pigeon pea is that 35 (41%) and 16 (34%) of the released cultivars in chickpea and in pigeonpea, respectively, were developed in India involving one or two genotypes as one of the ancestors in their pedigree [24]. A study was conducted to compare the level of genetic diversity in pulses and concluded that the range of genetic diversity has been found to be narrow among both the cultivated and wild relatives of pigeon pea and lentil as compared to other pulses [2].

4. Conclusion

On the basis of results based on present study and previous studies on pigeonpea we can conclude that there is an urgent need to incorporate diverse parents in breeding programmes of pigeonpea and generate more DNA markers which can be useful in molecular breeding programmes of this crop.

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