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RAPD Analysis in cowpea [*Vigna ungaiculata* (L.) WALP]

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The present investigation was carried out with 18 germplasm of cowpea. Purified and isolated DNA was subjected to PCR based marker (RAPD) for assessment of genetic diversity. The quality of DNA was determined by calculating ratio between A260 and A28o. The ratio between A260 and A280 was observed 1.41-2.01 which indicated a moderately good quality of plant DNA. The concentration of DNA ranged from 3.12mg/ml to 3.92 mg/ml in RAPD analysis. PCR (RAPD) involving 15 randomly selected decamer primers, of which only 9 primers gave good amplified product with template DNA. A total of 204 amplified fragments were formed by 9 primers. A total of 48 amplicon were obtained with 9 primers with an average of 3.2 bands per primer. Out of 48 scorable bands, all 48 bends were polymorphic and the level of polymorphism was 100 per cent. From RAPD profiling similarty matrix was obtained and similarity coefficient ranged between 0.00 - 0.47. The dendrogram clearly divided the 18 cultivars into 5 main clusters. Cluster I includes 13 genotypes of cow pea and cluster II includes only one cultivar. Cluster III includes 2 genotypes. Cluster IV and V includes 1 genotype each. On this basis of similarty matrix, dendrogram was constructed using UPGMA method.

Key words : RAPD analysis, Cowpea, Molecular marker

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INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp] a grainlegume is also referred as southern pea, black eye pea, crowder pea, lubia and frijole. It is a valuable crop because of its high protein content (23-29%) and nutritive value (Ehlers and Hall, 1997). Cowpea has ability to fix atmospheric nitrogen. Cowpea establishes symbiotic association with rhizobium body bacteria enabling it to fix atmospheric nitrogen. It is an important source of proteins for vegetarians or those who cannot afford protein rich foods like fish, meat and eggs. It has been referred to as poor man's meat. The proteins in cowpea seed are rich in essential amino acids such as lysine and tryptophan as compared to cereal grains. Young leaves, pods and peas of cowpea are rich in vitamins and minerals. It can also be used as green manure crop and livestock fodder. Cowpea can be intercropped with cereal crops like millet and sorghum. It is an annual autogamous crop. It belongs to family *Leguminoeceae* with chromosome number 2n =22.

Cowpea (*Vigna unguiculata* L.) is one of the important *Kharif* pulse crop grown in India. It is warm season crop, well adapted to many areas of the tropical and subtropical zones. Cowpea is tolerant to heat and dry conditions, but is intolerant to frost. Drought resistance is one reason that cowpea is an important crop in many under developed parts of the world. The crop is sown from March to April and is harvested between June and July depending upon its end use. Incorporation of cowpea as a legume in crop sequences enriches soil fertility and provides a dense soil cover to check wind erosion and evaporation loss of soil water.

Molecular markers have been proved for valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. Different markers might reveal different classes of variation (Powell *et al.,* 1996; Russell *et al.,* 1997). It is correlated with the genome fraction surveyed by each kind of marker, their distribution throughout the genome and the extent of the DNA target which is analyzed by each specific assay (Davila *et al.,* 1999).

RAPD markers are commonly used because they are quick and simple to obtain enabling genetic diversity analysis in several types of plant material such as natural populations, population in breeding programmes and germplasm collections (Ferreira and Grattupaglia,

1996).RAPD markers are superior when simplicity and cost is considered (William *et al.,* 1990). RAPD has been used in analysis of genetic distance in different plant species (Lashermes *et al.,* 1996; Samec and Nasinec, 1996; Colombo *et al.,* 2000).

RESEARCH METHODOLOGY

The present investigation was conducted on leaves collected from cowpea germplasm grown in pots in the Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Udaipur. The seeds of 18 different varieties of cowpea were obtained from the Department of Plant Breeding and Genetics, Rajasthan College of Agriculture, Udaipur. Seeds were sterilized with 1 per cent of $HgCl_2$ solution before sowing. Seedlings were grown in pots. Leaves were collected at 25 days after sowing.

Chemicals:

All chemicals used in DNA Isolation and PCR (Polymerase Chain Reaction) were of analytical grade. They were purchased from the firms like Himedia Laboratory, SRL and Banglore Genei Pvt. Ltd., Bangalore, India.

DNA isolation, purification and quantification:

The total 5 gram of leaf tissues was collected from a single pot of each germplasm. The leaf tissue was frozen in liquid nitrogen and ground to a fine powder in a pestle and mortar. The genomic DNA was isolated from powdered leaf tissue using the CTAB method described by Doyle and Doyle (1990) and treated with RNase to eliminate RNA. DNA concentration was measured by UV -absorbance method. The concentration of DNA preparation varied from 3.12 µg/ml (CP-28) to 3.95 µg/ml (CP-2) as shown in Table 1. The integrity of the isolated DNA was verified by visualization of DNA on 0.8 per cent Agarose gel with DNA standard uncut lambda DNA. The quality of DNA was determined as the ratio of A_{260}/A_{280}, which ranged from 1.41 to 2.01, indicated to be a good quality plant DNA (Table 1).

List of primers:

A set of decanucleotide RAPD primers were used for PCR amplification. The sequences of these primers were selected from literature and purchased from Banglore Genei Pvt. Ltd. Bangalore. The details of primer code sequence of the primer and GC contents are given in Table 2.

RAPD Analysis:

Random amplified polymorphic DNA (RAPD) was done by using 15 random primers obtained from Banglore Genei Pvt. Ltd. Banglore. PCR reactions were performed in final volume of 25 ml containing 10 x assay buffer 1 units of Taq. DNA polymerase, 250 mM each of dNTPs,

Table 1: Concentration of DNA in cowpea (*Vigna unguiculata* L.) germplasm

Sr. No.	Germplasm name	Code No.	OD at A_{260} nm	OD at A_{280} nm	Ratio of A_{260}/A_{280}	Conc. of DNA (µg/µl)
1.	CP-2	V1	0.395	0.219	1.80	3.95
2.	CP-3	V2	0.313	0.185	1.69	3.13
3.	CP-4	V3	0.362	0.204	1.77	3.62
4.	CP-5	V4	0.361	0.201	1.79	3.61
5.	CP-7	V5	0.361	0.205	1.76	3.61
6.	CP-9	V6	0.315	0.156	2.01	3.15
7.	CP-10	V7	0.362	0.214	1.69	3.62
8.	CP-19	V8	0.385	0.206	1.86	3.85
9.	CP-20	V9	0.380	0.199	1.90	3.80
10.	CP-21	V10	0.361	0.212	1.70	3.61
11.	CP-22	V11	0.315	0.207	1.52	3.15
12.	CP-23	V12	0.318	0.214	1.48	3.18
13.	CP-24	V13	0.356	0.212	1.67	3.56
14.	CP-25	V14	0.344	0.207	1.66	3.44
15.	CP-26	V15	0.392	0.214	1.83	3.92
16.	CP-27	V16	0.387	0.274	1.41	3.87
17.	CP-28	V17	0.312	0.208	1.50	3.12
18.	CP-29	V18	0.371	0.202	1.83	3.71

10 pmols/reaction of random primer's and 25 ng of tempelate DNA. The polymerase chain reaction (PCR) was performed in PCR machine (Thermocycler) using the following cycling parameters:

PCR reaction cycle						
Cycle	Denaturation		Annealing		Extension	
First cycle	94°C	3 min	-	-	-	-
45 cycle	94°C	1 min	36°C	1 min	72°C	2min
Last cycle	-	-	-	-	72°C	2min

Following the amplification, the PCR products were loaded on 1.6 %Agarose gel (himedia) which was prepared in 1 x T AE buffer containing 0.5 µg/ml of the ethidium bromide. The amplified products were electrophoresed for 2.5-3 hrs at 50 V with cooling. After separation the gel was viewed under UV transilluminator and photographed with the help of gel documentation system (Alpha DG DOC).

Scoring the RAPD products:

In order to score and preserve banding pattern photograph of the gel was taken by a Gel Documentation System, under UV transilluminator. RAPD bands were designated on the basis of their molecular sizes (length of polynucleotide amplified). 100bp-3000bp DNA ladder loaded simultaneously with primer products in the gel was used to estimate the molecular sizes. The distance run by amplified fragments from the well was translated to molecular sizes with reference to molecular weight marker. The presence of each band was scored as '1' and its absence as '0'. Faintly visible bands were not scored, but a major band corresponding to faint bands was considered for scoring.

Statistical analysis for similarity coefficient:

The scores (0 or 1) for each band obtained from photograph were entered in the form of a rectangular data matrix (qualitative data matrix). The pair-wise association coefficients were calculated from qualitative data matrix using Jaccard's similarity coefficient. The equation for calculating Jaccard's similarity coefficients 'F' between two samples A and B is:

$$F = n_{xy} / (n_l - n_z)$$

n_{xy} = Number of bands common to sample A and sample B.

n_l = Total number of bands present in all samples.

n_z = Number of bands not present in sample A or B but found in other samples.

Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationships of the clones using computer program NTSYS pc version 2.02 (Rohlf, 1998).

RESULTS AND ANALYSIS

The results obtained from the present investigation are presented below:

RAPD analysis:

Fifteen random decamer primers obtained from (Banglore Genei Pvt. Ltd., Banglore) having high per cent of (G+C) contents were used for RAPD analysis in 18 different genotypes of cowpea for detecting polymorphism. The primer codes and their arbitrary sequences are shown in Table 2. Out of 15 primers screened, 9 primers produced amplification, while 6 primers did not show amplification in any of the genotype. These 9 primers showed 100 per cent polymorphism. The DNA amplification and polymorphism generated among various genotypes of cowpea using random primers is presented in Table 3.

For each primer, RAPD fragments were scored on the basis of presence or absence of amplified products in the genotypes. Presence of band in one genotype was

Table 2: Detail of RAPD primers used in molecular analysis of cowpea (*Vigna unguiculata* L.) germplasm

Sr. No.	Primer*	Sequence 5' to 3'	G:C content (%)
1.	OPA-01	CAGGCCCTTC	70
2.	OPA-02	TGCCGAGCTG	70
3.	OPA-03	AGTCAGCCAC	60
4.	OPA-04	AATCGGGCTG	60
5.	OPA-05	AGGGGTCTTG	60
6.	OPB-01	GTTTCGCTCC	60
7.	OPB-02	TGATCCCTGG	60
8.	OPB-03	CATCCCCCTG	70
9.	OPB-04	GGACTGGAGT	60
10.	OPB-05	TGCGCCCTTC	70
11.	OPB-06	TGCTCTGCCC	70
12.	OPB-07	GGTGACGCAG	70
13.	OPB-08	GTCCACACGG	70
14.	OPB-09	TGGGGGACTC	70
15.	OPB-10	CTGCTGGGAC	70

*Operon series code

Table 3: Polymorphism information of RAPD primers analyzed

Sr. No.	Primers code	Sequences (5' →3')	Total no. of bands (a)	Total no. of polymorphic bands (b)	Polymorphism % (b/a x 100)
1.	OPA-01	CAGGCCCTTC	4	4	100
2.	OP A-02	TGCCGAGCTG	NA	NA	NA
3.	OP A-03	AGTCAGCCAC	NA	NA	NA
4.	OP A-04	AATCGGGCTG	NA	NA	NA
5.	OP A-05	AGGGGTCTTG	NA	NA	NA
6.	OPB-01	GTTTCGCTCC	5	5	100
7.	OPB-02	TGATCCCTGG	5	5	100
8.	OPB-03	CATCCCCCTG	NA	NA	NA
9.	OPB-04	GGACTGGAGT	NA	NA	NA
10.	OPB-05	TGCGCCCTTC	8	8	100
11.	OPB-06	TGCTCTGCCC	5	5	100
12.	OPB-07	GGTGACGCAG	7	7	100
13.	OPB-08	GTCCACACGG	5	5	100
14.	OPB-09	TGGGGGACTC	6	6	100
15.	OPB-10	CTGCTGGGAC	3	3	100
		Total	48	48	-
		Average	3.2	3.2	100

NA-Not amplified

taken or denoted by one (1) and the absence of band was taken as zero (0). In this way, banding pattern for all the 18 genotypes were obtained for all the primers showing amplification.

Primer numbers OPA-01, OPB-01, OPB-01, OPB-02, OPB-05, OPB-06, OPB-07, OPB-08, OPB-09 and OPB-10 gave different bands on agarose gel. Molecular size of bands ranged between 100bp to 3000bp. In all 204 clear and reproducible bands were obtained from 9 primers, all of these were polymorphic.

Out of fifteen primers OPB-05 gave the highest number of bands *i.e.* 8 followed by OPB-07 and OPB-09 which gave 7 and 6 bands, respectively. Primers OPB-08, OPB-06, OPB-02 and OPB-01 gave 5 bands each on agarose gel. Primers OPA-01 and OPB-10 gave 4 and 3 bands, respectively. The number of DNA amplified fragments per primer ranged from 3 (OPB-10) to 8 (OPB-05). The most informative primers were OPB-05 OPB-07 and OPB-09 with 8, 7 and 6 polymorphic bands, respectively.

The maximum number of amplified band was found in primers OPB-07 and OPB-05 which produced 41 and 40 bands, respectively, while primer OPA-01 and OPB-02 produced only 6 and 15 bands.

The high level of polymorphism (100%) observed during present study agreed with the results of previous studies carried out in mungbean [*Vigna radiata* (L.)] by Saini *et al.* (2010) who reported a total of 411 RAPD amplicons, of which 382 (92.9%) were polymorphic. The per cent polymorphism ranged from 42.85 to 100 per cent. Similarly Choudhury *et al.* (2008) observed a total of 796 amplified products using RAPD, of which 587 showed polymorphism (73.7 %) in pigeonpea [*Cajanus cajan* (L.)] cultivars.

Khan *et al.* (2000) studied genetic diversity among 31 species using RAPD markers in three subspecies and one interspecific hybrid of cotton *(Gossypium).* A total of 579 amplified bands were observed, with 12.9 bands per primer of which 99.8 per cent were polymorphic. Similarly Kutty *et al.* (2006) reported the genetic diversity among 24 cultivars of short day onions using RAPD markers. They used 90 arbitrary decamer primers out of which 15 primers were selected which yielded 137 bands, 91.24 per cent of which were polymorphic. None of the primers produced a unique banding pattern for each cultivar.

Rana and Bhat (2005) used RAPD markers for genetic diversity estimation in 59 cotton cultivars belonging to four cultivated species of cotton. The selected eighteen RAPD primers produced a total of 251 amplicons, which generated 97.21 per cent polymorphism which corroborate well with those reported in present study.

Genetic similarity of cowpea genotypes was estimated by a similarity matrix obtained by using NTSYSpc programme. Out of total number of 48 bands, all the bands were found to be polymorphic and hence,

Table 4: Jaccard's average similarity coefficient for 18 cowpea *(Vigna unguiculata* L.) genotypes of based on RAPD profiling

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V15	V15	V16	V17	V18
1.	1.00																	
2.	0.37	1.00																
3.	0.18	0.20	1.00															
4.	0.43	0.21	0.07	1.00														
5.	0.40	0.25	0.17	0.23	1.00													
6.	0.24	0.08	0.05	0.41	0.07	1.00												
7.	0.19	0.21	0.10	0.17	0.12	0.17	1.00											
8.	0.11	0.18	0.00	0.07	0.07	0.05	0.00	1.00										
9.	0.00	0.08	0.00	0.11	0.07	0.05	0.20	0.00	1.00									
10.	0.17	0.27	0.18	0.24	0.24	0.12	0.29	0.00	0.17	1.00								
11.	0.35	0.17	0.05	0.42	0.26	0.28	0.08	0.16	0.10	0.17	1.00							
12.	0.29	0.22	0.05	0.45	0.15	0.23	0.17	0.15	0.05	0.21	0.42	1.00						
13.	0.28	0.14	0.13	0.32	0.13	0.31	0.21	0.19	0.12	0.39	0.38	0.31	1.00					
14.	0.17	0.12	0.18	0.29	0.11	0.16	0.29	0.08	0.27	0.43	0.27	0.26	0.47	1.00				
15.	0.23	0.12	0.08	0.29	0.24	0.16	0.29	0.08	0.17	0.43	0.22	0.21	0.32	0.43	1.00			
16.	0.00	0.00	0.00	0.06	0.00	0.12	0.06	0.08	0.17	0.18	0.08	0.04	0.14	0.18	0.18	1.00		
17.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	
18.	0.16	0.11	0.07	0.23	0.10	0.29	0.25	0.07	0.07	0.22	0.20	0.19	0.29	0.22	0.10	0.10	0.00	1.00

level of average polymorphism was 100 per cent (Table 3). Average number of bands per primer was 3.2 and the average number of polymorphic bands was found to be 3.2 per primer.

Genetic relationship among the germplasm and cluster analysis:

The banding pattern generated and polymorphism reflected in these patterns was used to calculate the diversity among accessions taken for present study. Genetic similarity estimation based on RAPD banding patterns was calculated using method of Jaccard's coefficient analysis. The similarity coefficient matrix generated for the primers was subjected to algorithm UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and clusters were generated using NTSYSpc 2.02 program (Rohlf, 1998). The dendrogram showing relationships among various varieties was constructed using these clusters (Fig. 1).

The RAPD data was used to obtain a similarity matrix (Table 4).The similarity coefficient for different genotypes lies in the range of 0.00 to 0.47. The average similarity across all the genotypes was found to be 0.24 indicating a moderate level of genetic similarity among the genotypes. The minimum similarity (0.00%) was observed between genotypes V3 and V8 (CP-4 and CP-19), V7 and V8 (CP-1O and CP-19), V1 and V9 (CP-2 and CP-20), V3 and V9 (CP-4 and CP-20), V8 and V9 (CPI9 and CP-20), V8 and V10 (CP-19 and CP-21), V1 and V16 (CP-2 and CP-27), V2 and V16 (CP-3 and CP-27), V3 and V16 (CP-4 and CP27), V5 and V16 (CP-7 and CP-27). V17 (CP-28) have also minimum similarity (0.00%) to all other genotypes. The maximum similarity (0.47%) was observed between V13 and V14 (CP-24 and CP-25).

Vafaie-Tabar *et al.* (2003) used RAPD markers and morphological characteristics for evaluation of genetic diversity among 7 diploid *(G. arboreum)* and 15 tetraploid *(G. hirsutum)* cotton cultivars. A total of 371 bands were amplified, 325 (87%) of which were polymorphic. The genetic variability detected among the cultivars using morphological characters was low (0.61) than that detected with RAPD (0.88). Similarly Ghalmi *et al.* (2010) analyzed genetic diversity in Algerian cowpea *(Vigna unguiculata* (L.) Walp.) by using 11 random amplified polymorphic DNA (RAPD). The eleven RAPD primers yielded 77 bands, of which 45 (58.44%) were polymorphic; the genetic similarity ranged from 66.0 to 96.7 per cent.

Pathak *et al.* (2010) performed RAPD analysis using 10 decamer arbitrary primers. Out of 10 random primers, five primers (OPA-16, OPP-7, OPB-12, OPP-9 and OPA-14) were selected for the analysis. These five selected primers generated a total of 49 amplicons

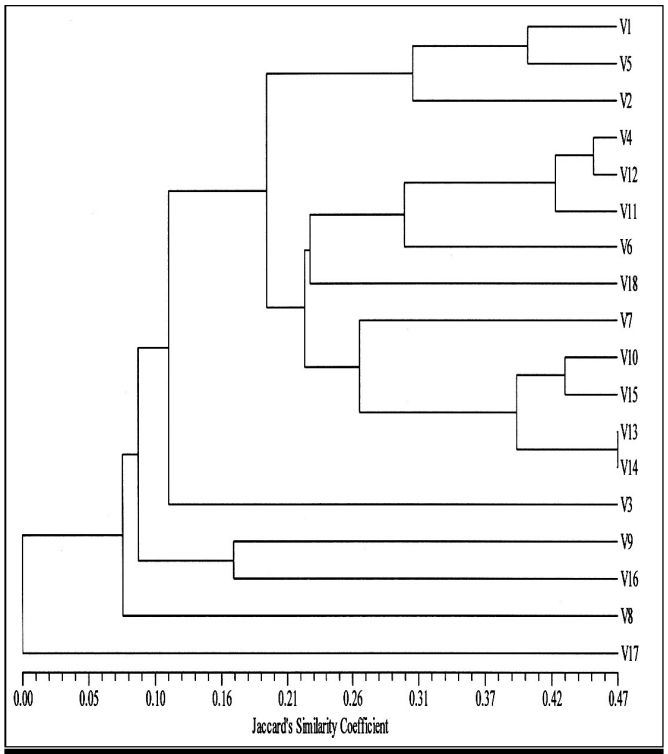

Fig 1: Jaccard's average similarly coefficient among cowpea (*Vigna unguiculata* L.) genotypes based on RAPD prefiling

of which 39 were polymorphic and exhibited high degree of marker index ranging from 66.6 to 87.5 per cent polymorphism in banding pattern.

Dendrogram:

A dendrogram was constructed using similarity matrix value as determined from RAPD data for 18 genotypes using UPGMA (unweighted pair group method of arithmetic averages) subprogramme of NTSYSpc programme. The dendrogram constructed depicts the relationship among 18 genotypes of cowpea used in present study. The dendrogram generated on the basis of Jaccard's similarity coefficient, clearly indicated five main clusters. The cluster I which was the major cluster included 13 genotypes *viz.* V1 (CP-2), V5 (CP-7), V2 (CP-3), V4 (CP-5), V12 (CP-23), V11 (CP-22), V6 (CP-9), V18 (CP-29), V7 (CP-10), VIO (CP-21), V15 (CP-26), V13 (CP-24) and V14 (CP-25) with similarity coefficient of 0.19 with other four main clusters.

The cluster I was clearly divided into two subgroups, subgroup A and subgroup B. Subgroup A included 3 genotypes *i.e.* V1 (CP-2), V5 (CP-7) and V2 (CP-3). In subgroup A genotype V1 (CP-2)-V5 (CP-7) were related to each other at similarity coefficient of 0.40. Genotype V2 (CP-3) joined with [V1 (CP-2)- V5 (CP-7)] at similarity coefficient 0.31.

Subgroup B included 10 genotypes *i.e.* V4 (CP-5), V12 (CP-23), V11 (CP-22), V6 (CP-9), V18 (CP-29), V7 (CP-10), V10 (CP-21), V15 (CP-26), V13 (CP-24) and V14 (CP-25) with similarity coefficient 0.22. In this subgroup the maximum similarity was observed between genotypes V13 (CP-24)-V14 (CP-25) with similarity coefficient 0.47. The other two pairs of genotypes which were closely related were [V4 (CP-5)-V12 (CP-23)] and [V10 (CP21)-V15 (CP-26)] with similarity coefficients 0.45 and 0.43, respectively. Genotype V11 (CP-22) joined the subgroup V4 (CP-5)-V12 (CP-23) at similarity coefficient 0.43. The genotype V6 (CP-9) joined the subgroup comprising genotypes V4, V12 and V11 at similarity coefficient of 0.30. Genotype V18 (CP-29) was related to sub cluster [V4, V12, V11 and V6] at similarity coefficient 0.23. Cluster V10-V15 and V13-V14 were related to each other at similarity coefficient 0.40 which indicated that these four genotypes were genetically close. Genotype V7 (CP-10) joined the sub cluster comprising genotypes (V10, V15, V13 and V14) at similarity coefficient of 0.27.

The cluster II included only one genotype *i.e.* V3 (CP-4) which showed the similarity coefficient of 0.11 with cluster J. The cluster III included genotypes V9 (CP-20)-V16 (CP-27) with similarity coefficient of 0.17. Cluster III joined cluster II at similarity coefficient of 0.09. The cluster IV which has only one genotype *i.e.* V8 (CP-19) showed similarity coefficient of 0.075 with other three main clusters. The cluster V also has single genotype *i.e.* V17 (CP-18) which showed the least similarity coefficient *i.e.* 0.00 with other four main clusters.

The cluster tree, in a way, tells same story as revealed by similarity matrix of 18 genotypes. The association amongst different genotypes is presented in the form of dendrogram, the genotypes which are lying close to each other in the dendrogram are genetically closer to each other than those lying apart.

Hence, study comprising 18 cowpea verities shows that there is an association between similarity matrix and the dendrogram obtained by RAPD marker. The understanding of distribution of genetic variation using RAPD analysis will help in identification of superior genotype for cultivar upgradation as well as to evolve breeding strategies for genetic improvement in cowpea. Nevertheless, it could be concluded that RAPD profiles were more efficient in detecting polymorphism in cowpea

verities.

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