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RAPD Analysis for Genetic Diversity and Verification of Hybridity in Cowpea [*Vigna unguiculata* (L.) Walp.]

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A B S T R A C T

Cowpea (*Vigna unguiculata*) is a legume crop growing in almost all parts of world including tropics and subtropics. This study was thus undertaken to assess genetic diversity among the different parents used for hybridization and confirmation of hybrids of cowpea (*Vigna unguiculata*) *by* using randomly amplified polymorphic DNA (RAPD) markers. RAPD profiles for 11 genotypes were generated with 20 random decamer primers. Out of 20 primers screened 15 primers gave scorable DNA fragments and each of the 15 primers revealed various levels of polymorphism. These primers generated 476 DNA fragments in the average range of 167bp to 3300bp, of which 367 were polymorphic. The percentage of polymorphism ranged from 35.29 to 100% with an average of 79.96%. The overall range of similarity among 11 genotypes was found to be very wide, ranging from 0.328 to 0.613 which indicates there was high variability among the cowpea genotypes under study. The RAPD primer OPA-18 was effectively used for confirmation of hybridity.

Introduction

Cowpea (*Vigna unguiculata*) cowpea, is a highly variable legume crop that originated in Africa. It is mainly grown for grain, forage and green manure. Its grain is rich in protein and digestible carbohydrates and its energy content is nearly equal to that of cereal grains. The genetic variability for different characters are at most importance in selecting the desired genotypes for any breeding programme. Information on genetic variability among different characters is essential for systematic breeding programme. Several different

methods for documenting genetic information are isozyme analysis, restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD). The RAPD technique employs 8-10 base pair random primers to locate random segments of genomic DNA to reveal polymorphisms. It is a simple, reliable and relatively straight-forward technique to apply, and the number of loci that can be examined is unlimited. RAPD analysis is viewed as having a number of advantages over RFLPs and other techniques. The ease and simplicity of the RAPD technique make it ideal for genetic mapping in plant and animal breeding programs, and DNA fingerprinting, with particular utility in the field of population genetics. RAPD analysis would be very useful in breeding for rapid and easy verification of hybridity in large seedling population and even purity testing of different seed lots of pulses and cereals.

Materials and Methods

Plant material

For the present experimental study 11 genotypes of cowpea (*Vigna unguiculata* (L.) Walp.) selected from the germplasm collected from Rajasthan College of Agriculture, Udaipur(Raj) and Department of Agricultural Botany, Dapoli (MS) the research farm of the Department of Agricultural Botany, College of Agriculture, Dapoli. All the 11 genotypes of cowpea were grown in the field. The leaf samples for DNA isolation were collected from 15 days old seedlings.

DNA extraction

The genomic DNA was isolated from the young newly flushing leaves by following the protocol of Doyle and Doyle (1990) i.e. Rapid method. Purification of DNA was done to remove RNA, proteins and polysaccharides which were the major contaminants. RNA was removed by RNase treatment. RNase was added to the DNA sample @100 ug ml^{-1} and incubated at 37°C for 1 hr. Concentration of DNA in the sample was determined by agarose gel electrophoresis with standard DNA i.e., uncut lambda DNA on 0.8per cent agarose gel and by comparison of the intensity of staining with ethidium bromide.

RAPD analysis

PCR amplification reactions were performed with random decamer primers obtained from Operon Technology (Alamenda, USA) in an Eppendorf, Master cycler gradient (Hamburg Germany). A total of 20 RAPD primers were subsequently used for PCR amplification. For the RAPD analysis, initially the PCR master mix was standardized for each PCR component and the optimum concentration of each component in master mix which gave better amplification was used for further work. PCR reaction was performed in 10 µl reaction mixture consisting 3U TaqDNA polymerase (Banglore Genei Ltd.), 1.25µl10x Taq assay buffer with 0.25µl $MgCl_2$, 10 mMd NTPs 0.50µl, 25 pmoles of random decamer primer 0.50µl and 30-50ng of template DNA 1.0µl. The amplification profile for RAPD consisted of initial denaturation at 94^0C for 5 min, followed by 35 cycles comprising of a denaturation step at 94^0C for 30 sec, an annealing step at 37 ^{0}C for 1 min and an extension step at 72 ^{0}C for 30 sec. The cycling program was terminated by a final extension step at 72 ^{0}C for 7 min. The amplified products in RAPD reaction were separated by electrophoresis in 2 per cent agarose gel (SRL, India), containing ethidium bromide in 1x TAE Buffer (pH 8.0) and separation was carried-out by applying constant voltage of 80 volts for 1 hr. The standard DNA ladder used was Φ x174/Hae III digest. PCR and gel electrophoresis were carried out two times and only reproducible

patterns were used for data analysis. The gels were photographed under UV light using Pentax K 312 nm camera. The images of gels were also taken by the documentation systems (Uvi-Tech. Fire reader, Cambridge, England) and saved in computer for further analysis.

Data analysis

RAPD markers across the 11genotypes were scored for their presence (1) or absence (0) of bands for each primer. By comparing the banding patterns of genotypes for a specific primer, genotype-specific bands were identified and faint or unclear bands were not considered. The binary data so generated was used to estimate the levels of polymorphism by dividing the number of polymorphic bands by the total number of scored bands. Pair-wise similarity matrices were generated by Jaccard's coefficient of similarity by using MVSP-A Multivariate Statistical Package - 5785 (Version 3.1). The cluster analysis was performed from the distance matrix using Jaccard's similarity coefficients.

Results and Discussion

The RAPD profiles for all 11 cowpea parents were generated with 20 random decamer primers out of which15 primers gave scorable DNA fragments. A total of 476 amplified products were observed out of which 367 were polymorphic. The number of amplified DNA fragments for each primer varied from 20 to 51, with an average of 31.73 fragments per primer. The highest number of fragments (i.e. fifty-one) was generated by primer OPA-13 while OPA-18 generates 50 DNA fragments with 100% polymorphism. The percentage of polymorphism ranged from 35.29 to 100% with an average of 79.96%. (Table 1) Size of the amplified products ranged from 167 bp to 3300 bp. The primers OPA-02, OPA-03, OPA-10, OPA-11, OPA-16 and OPA-18 produced distinct RAPD patterns (100% polymorphism) for all the 11 parents. The three primers OPA-07(86%), OPA-02 and OPA-18 (80%) had the highest polymorphic information content value. Similar results have also been reported by Phansak *et al.,* (2005) Patil *et al*., (2013) Fall *et al*., 2003. The percentage of polymorphism across the cowpea genotypes ranged from 35.29-100 per cent. Karuppanapandian *et al*., (2006) recorded similar results in cowpea landraces (25-100%). The monomorphic fragments were constant bands and cannot be used to study diversity while polymorphic fragments reveal differences and can be used to examine and establish systematic relationship among the genotypes. The high genetic diversity detected in the cowpea genotypes analysed, probably indicated that cultivars were originally generated by different ancestors of cowpea in the past.

The similarity co-efficient ranged from 0.328 (between genotype CPD-220 and CPD-25, CPD-31 and CPD-25) to 0.613 (between genotype GS-9240 and NKO-32) indicated the distinctness (Table 2) which indicates there was high variability among the cowpea genotypes under study. These genotypes could be useful in future breeding programmes. Previous studies using allenozymes, RAPD and microsatellites, indicated a low level of variation in cowpea reported by Li *et al.,* (2001); Pasquet, (2000); Tosti and Negri, (2002), while Patil *et al*., (2013) and Anatala *et al*., (2014), reported high level of variation among the genotypes studied. The genotypes CPD-220, CPD-25, CPD-31 and CPD-25 showed very minimum differences between them at genotypic level.

The cluster analysis based on the similarity co-efficient clearly distinguished all the 11 genotypes into two groups. The first cluster included only single genotypeCPD-25and was most diverse from rest of the 10 genotypes (Fig. 1). The second cluster further subdivided into two subclasses (Fig. 2–6).

Table.1 Primer wise amplification and percent polymorphism of Cowpea genotypes.

Sr No	Name of Primer	Number of fragments	Number of monomorphic fragments	Number of polymorphic fragments	Polymorphism percentage	PIC value	Range of amplification
1	OPA 02	20	0	20	100	0.80	947 – 3300
2	OPA 03	36	0	36	100	0.77	399 – 1210
3	OPA 04	31	11	20	64.52	0.45	415 – 808
4	OPA 05	32	11	21	65.63	0.50	822 – 1959
5	OPA 07	37	0	37	100	0.86	461 – 1900
6	OPA 08	31	0	31	100	0.68	439 – 1422
7	OPA 09	31	11	20	64.52	0.45	637 – 1991
8	OPA 10	18	0	18	100	0.44	492 – 738
9	OPA 11	17	0	17	100	0.41	555 – 1802
10	OPA 12	45	22	23	51.11	0.52	167 – 1747
11	OPA 13	51	33	18	35.29	0.38	863 – 1748
12	OPA 15	28	11	17	60.71	0.35	674 – 1794
13	OPA 16	24	0	24	100	0.62	773 – 2108
14	OPA 18	50	0	50	100	0.80	395 – 1798
15	OPA 19	25	11	15	57.69	0.27	610 – 1601
	Total	**476**	**110**	**367**	**1199.47**	**8.3**	
	Average	**31.73**	**7.33**	**24.47**	**79.96**	**0.55**	

Table.2 Genetic distances based on RAPDs pooled over the 15 primers in cowpea parents

	PCP 97102	GS 9240	NKO 32	CPD 219	CPD 220	CPD 172	CPD 31	CPD 193	CPD 173	CPD 25	CPD 83
PCP 97102	1.000										
GS 9240	0.500	1.000									
NKO 32	0.565	0.613	1.000								
CPD 219	0.475	0.429	0.567	1.000							
CPD 220	0.492	0.400	0.532	0.518	1.000						
CPD 172	0.500	0.453	0.590	0.526	0.443	1.000					
CPD 31	0.467	0.492	0.583	0.574	0.483	0.492	1.000				
CPD 193	0.518	0.443	0.438	0.414	0.407	0.417	0.456	1.000			
CPD 173	0.600	0.444	0.557	0.545	0.536	0.517	0.564	0.431	1.000		
CPD 25	0.411	0.439	0.344	0.357	0.328	0.362	0.328	0.396	0.400	1.000	
CPD 83	0.526	0.385	0.424	0.377	0.491	0.359	0.466	0.491	0.491	0.407	1.000

Fig.1 Dendrogram depicting 11 cowpea parents based on the genetic distances generated by 15 random primers

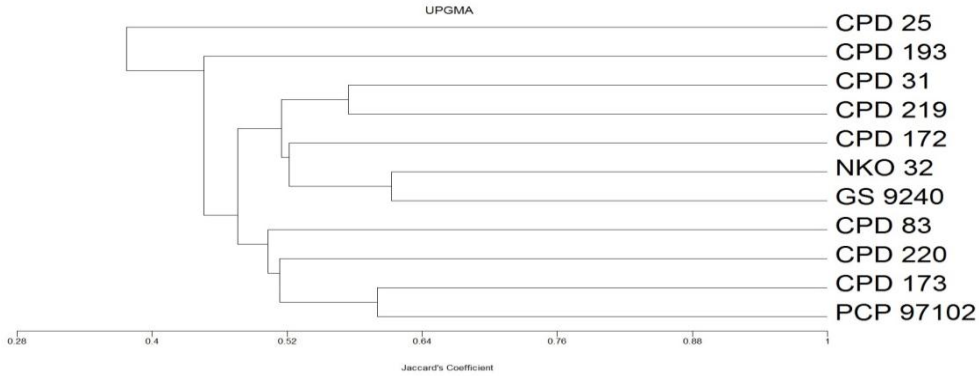


Fig.2 RAPD profile pattern of eleven cowpea parents using primer OPA-07

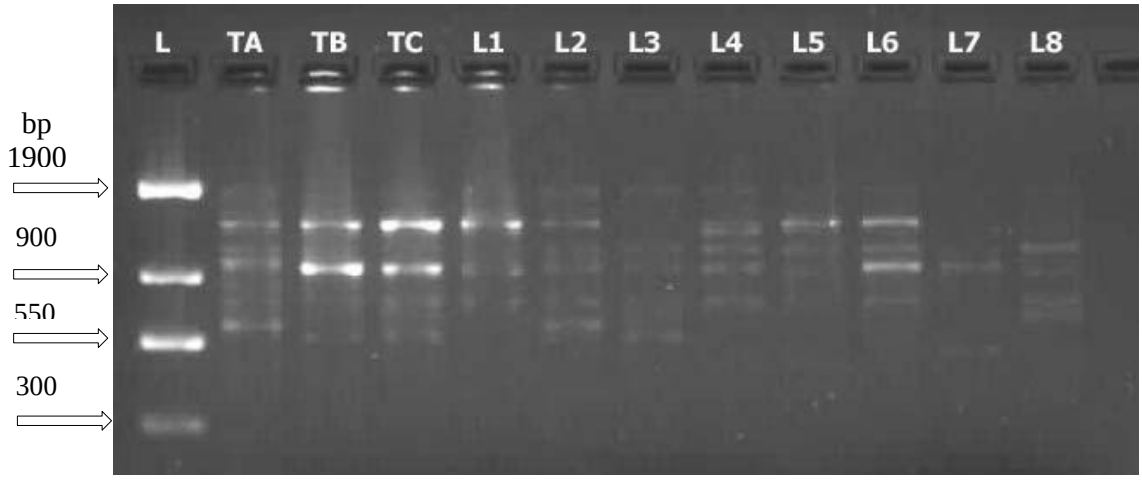


Fig.3 RAPD profile pattern of eleven cowpea parents using primer OPA-09

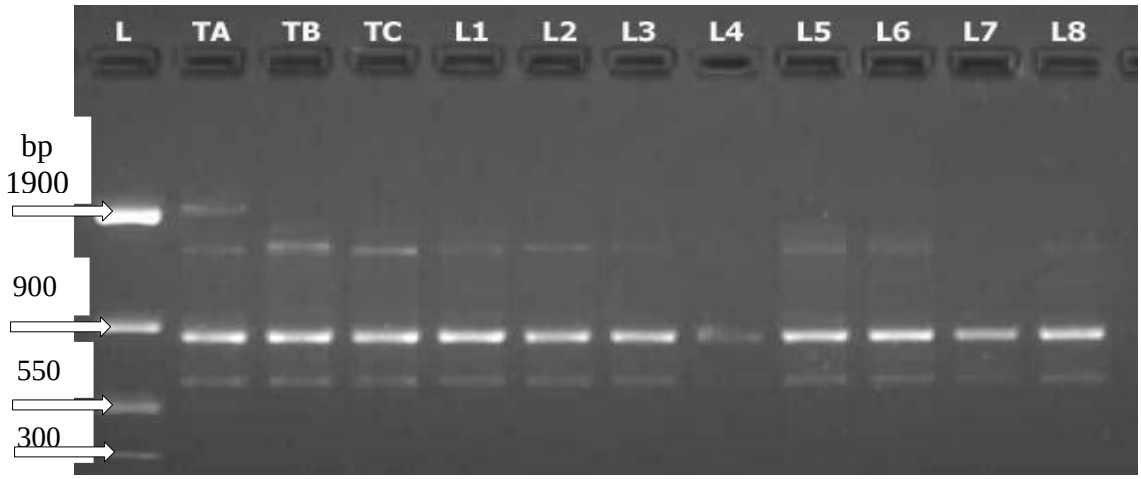


Fig.4 RAPD profile pattern of eleven cowpea parents using primer OPA-12

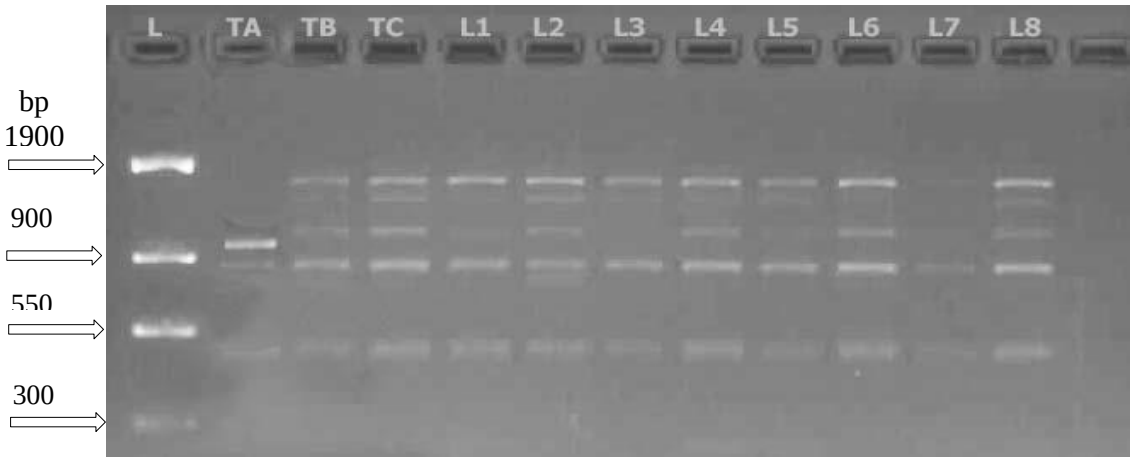


Fig.5 RAPD profile pattern of hybrid 13, 14 and 15 alongwith their parents- primer OPA-18

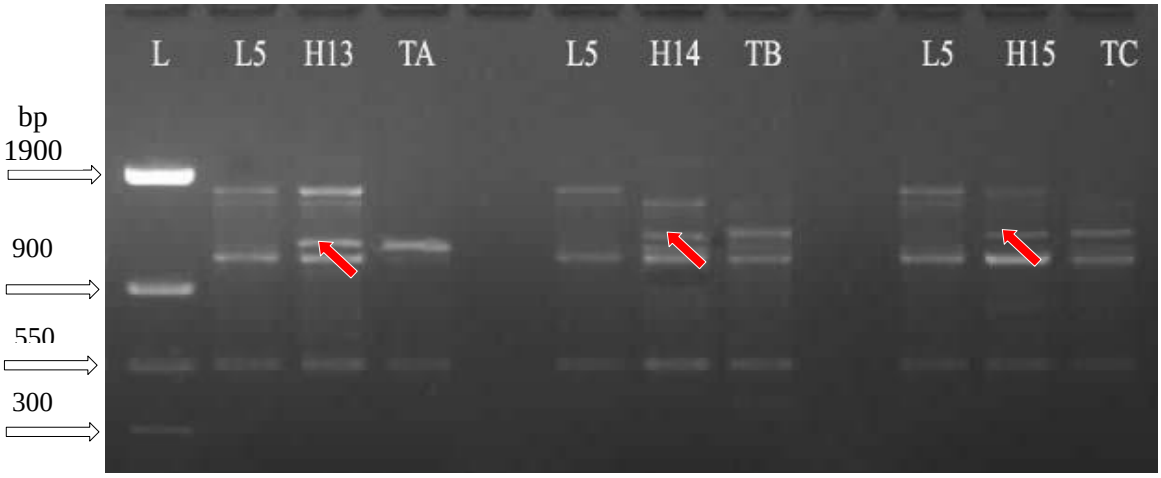
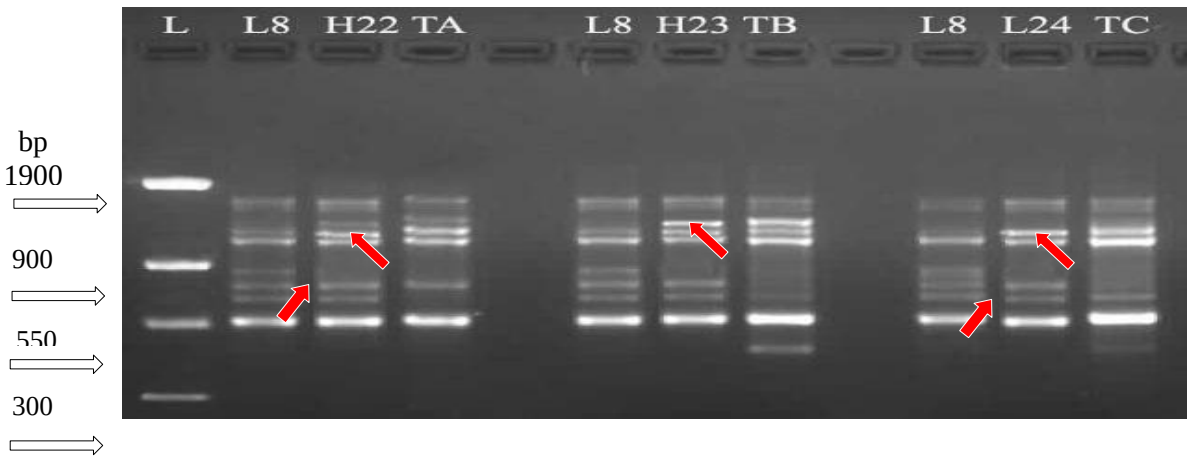


Fig.6 RAPD profile pattern of hybrid 22, 23 and 24 along with their parents- primer OPA-18



The first sub class of the second cluster included only one genotype i.e CPD- 193. The second sub class again subdivided into two sub class, the first sub class (IIBa) consists of 5 genotypes i.e. CPD-31, CPD-219, CPD-172, NKO-32, GS-9240 while the second sub class (IIBb) was formed with remaining 4 genotypes namely; CPD-83, CPD-220, CPD-173, PCP-97102.From the above clusters formed it was observed that, the genotype CPD-25 was more diverse from other 10 genotypes of cowpea. Patil *et al*., (2013) reported the RAPD profile of 30 cowpea genotypes grouped into two main clusters (I and II). The first cluster (I) was formed by the single genotype and the rest of 10 genotypes were included in to second cluster (II). Dendrogram revealed that the genotypes GS-9240 and NKO-32 were diverse and useful in future hybridization programme of cowpea.

The hybrid specific bands generated in RAPD analysis by primer OPA-18 was used for the identification particular F_1 hybrid of cowpea. The results obtained from RAPD fingerprinting were found to be useful to hybrid purity analysis, identification of hybrids and parentage confirmation. There is no previous report of utilization of RAPD markers for purity assessment of hybrids or hybrid identification in cowpea.

In conclusion, molecular markers can be exploited to generate the fingerprint database which can be utilized for analyzing the viability and purity of the seeds. This may result in the improvement of both, quality and quantity of crops. The RAPD technique appears to be the best alternative tool to assess genetic diversity, because it provides good discrimination in short time and at low cost. Critical analysis of the results obtained during present studies reveals that *Vigna unguiculata* genotypes could be efficiently characterized and classified using RAPD markers and by analyzing the banding pattern of the amplified products. The genotype GS-9240 and NKO-32 were diverse genotype while genotype CPD-25 observed as a unique genotype. These genotypes would be further exploited for *vigna* crop improvement. The RAPD primer OPA-18 was effectively used for confirmation of hybridity.

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