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RAPD BASED GENETIC DIVERSITY ASSESSMENT OF COWPEA

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Abstract

Genetic diversity of 16 cowpea genotypes was assessed through RAPD analysis. 25 random primers used in RAPD-PCR produced a total number of 160 amplified DNA bands with an average of 6.4 bands per primer. Out of total number of bands, 115 polymorphic and 45 monomorphic bands were amplified from the genome of 16 cowpea genotypes. The values of genetic similarity coefficient ranged from 0.544 to 0.825, indicating the presence of wide genetic diversity. Dendrogram constructed based on phylogenetic relationship analysis revealed that the highest genetic diversity (82.5%) found between genotype IC-559388 and IC-559390, while the lowest (54.4%) between the genotype EC-472283 and EC-528410. Clustering classified the 16 cowpea genotypes into 4 distinct clusters *i.e.* cluster A, B, C, D comprising of 6, 3, 4, 3 cowpea genotypes, respectively. These approaches will be useful for developing marker-assisted selection tools for genetic enhancement of the cowpea genotypes for desirable traits.

Key words: Cowpea, *Vigna unguiculata* L., RAPD, Polymorphism, cluster analysis and Genetic diversity

Introduction

Pulses are chief resource of vegetable proteins with necessary minerals and vitamins. Among the pulses, Cowpea is a food legume of significant cost-effective importance worldwide with high protein and mineral content. Cowpea (*Vigna unguiculata* L.) is an annual herb with strong tap root system and several spreading lateral roots in surface soil. It is a versatile crop, providing food for human and feed for live stock and it is a cash generating commodity for farmers, small and medium-size entrepreneurs. Fruits of cowpea are consumed at all stages of development such as green pods, fresh or dry seeds and young leaves are often used for soups and stews (Quaye *et al.,* 2009). In India, cowpea is cultivated in Maharashtra, Gujarat, Rajasthan, Punjab, Haryana, Madhya Pradesh, Kerala, Karnataka, Andhra Pradesh and Tamilnadu. It plays a critical role in the lives of millions of people in Africa and other parts of the developing world where it is a major source of dietary protein. These yield levels are very low and need to increase the yield by developing high yielding genotypes which are tolerant to biotic and abiotic stresses. Cowpea fixes nitrogen symbiotically and helps to restore the fertility of soil (Carsky *et al.*, 2002; Sanginga *et al.*, 2003).

The conventional process of crop breeding includes introduction, selection, hybridization and recombinations following pedigree, bulk or back cross techniques. Previous to the advancement of molecular markers, morphological markers were found to be helpful in varietal identification and assessing genetic diversity although they had certain restrictions. Later on protein based marker variants were used with limited success. They are stable and not influenced by environmental factors. It is well documented that the DNA markers have lots of advantages over the traditional morphological and biochemical markers. Among the DNA markers, polymerase chain reaction (PCR) based markers using arbitrary primers such as (Random Amplified Polymorphic DNA) RAPD, have been widely used for investigating genetic relatedness and diversity in crop population and cultivars.

Molecular markers have proven to be powerful tools in the estimation of genetic variation and in the illumination of genetic associations within and among the species. Various type molecular markers such as RFLP, RAPD and AFLP etc. are available which detect polymorphism

at the DNA level (Tosti and Negri, 2002; Fall *et al.*, 2003; Badiane *et al.*, 2004; Fana Sylla *et al.*, 2004; Diouf and Khidir, 2005; Karuppanapandian *et al.*, 2006). RAPD give high degree of polymorphism and thus help in differentiating even strongly associated cultivars (Williams *et al.*, 1990; Meena *et al.*, 2017). RAPD has been standardized and employed effectively by different workers (Xu *et al.*, 2000; Choudhury *et al.*, 2008; Bora *et al.*, 2016) to evaluate samples of various crops including *Vigna* species. For the simplicity and rapidity of the technique, RAPD technique has also been successfully employed for identification of genuineness of parents and their hybrids in many crop species (Santhy *et al.*, 2003; Ilbi *et al.*, 2004). The present study was undertaken to evaluate the genetic variability and relationship among different genotypes for genetic enhancement of cowpea using RAPD markers.

Material and Methods

Seeds of 16 cowpea genotypes were obtained from Scientific and Applied Research Centre (SARC), Meerut (UP) (table-1). The genetic material was grown in plot for germination and growth at Scientific and Applied Research Centre, Meerut with standard agronomical practices. Young new leaves of every one cowpea genotypes were collected separately and packed into polybags. Afterward, polybags for every one genotypes were freezing in liquid nitrogen and store up in deep freeze (-80°C) used for the separation of genomic DNA. Genomic DNA was isolated from young fresh leaves of 16 cowpea genotypes using the CTAB extraction method of Doyle and Doyle (1990) with minor modifications. Leaf material was ground to powder in liquid nitrogen and was then transferred to eppendorf tubes. 100mg leaf tissue was ground in 1ml CTAB extraction buffer (100mM Tris pH 8.0; 1.4M NaCl; 20mM EDTA pH 8.0; 0.2% (v/v) β-mercaptoethanol; 2 %(v/v) CTAB) and heated at 60°C for 30 min. DNA was isolated with one volume of a chloroform: isoamyl alcohol mix (24:1) and then centrifuged at 12000 rpm for 15 min at 4°C. Supernatant was taken and further mixed with one volume of chloroform: isoamyl. This was again centrifuged at 12000 rpm for 15 min at 4°C and then precipitated with isopropanol to 40% v/v final concentration. The DNA pellet was washed with 5mM ammonium acetate and 70% ethanol, dried and dissolved in 100µl of TE buffer (19mM Tris-HCl pH 8.0; 1mM EDTA pH 8.0). 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 µg ml^{-1} and incubated at 37°C for 1 hour.

25 RAPD primers were purchased from Integrated DNA Technology (IDT, USA) (table-2). All RAPD primers used in this study were dissolved in sterile TE buffer at a concentration of 15µg/ml. Polymerase chain reaction procedure were performed using 25ng genomic DNA with a final concentration of 25µl reaction volumes containing 10mM Tris-HCl pH 9.0; 50mM KCl; 0.1% TRITON X-100; 1.5mM MgCl; 0.1mM dNTP; 2mM primer; 0.5 unit of *Taq* DNA polymerase (Bangalore GeNei, India). Amplification were carried out in a thermal cycler programmed for 35 cycles with an initial melting at 94°C for 4 minute, followed by denaturation at 94°C for 1 minute. The annealing was performed at 37°C for 1 minute, which was then followed by polymerization at 72°C for 2 minute. Final extension step was at 72°C for 7 minute. Amplification products were subjected to horizontal electrophoresis unit on 1.8% agrose gel run in 1X TBE buffer at 50 volt for 2 hours and detected by staining using Ethidium Bromide. Standard molecular weight markers were used to determine the approximate size of amplification products. DNA bands were visualized on transilluminator and photographed by Gel documentation system.

Clear and stable bands amplified by RAPD primers were scored as 1 for present of bands and 0 for absent of bands. Polymorphism was calculated based on the presence or absence of bands. The data were entered in to MS-Excel data sheet and calculate the genetic distance using NTSYS-pc software (Rohlf, 2000). The dendrogram was constructed by using a distance matrix using Unweighted Pair Group Method of Arithmetic Mean (UPGMA) to access the genetic similarity and dissimilarity among all the genotypes. The percent polymorphism was calculated by using the following formula:

$$\text{Percent polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Results and Discussion

Polymorphism

RAPD technique is a simpler and faster method for characterization and analysis of genetic diversity among cowpea genotypes. Sixteen cowpea genotypes were analyzed for genetic diversity using 25 RAPD primers. All the primers produced polymorphic bands and revealed a high DNA polymorphism among the cowpea genotypes. A total of 160 bands were amplified among the cowpea genotypes through 25 RAPD primers, of which 115 bands were polymorphic showing high range of variability

Table 1: List of 16 cowpea genotypes

S. No.	Accessions	S. No.	Accessions
1.	IC-9883	**9.**	IC-402166
2.	IC-4506	**10.**	IC-402154
3.	IC-249141	**11.**	EC-472283
4.	IC-202786	**12.**	IC-559390
5.	IC-559399	**13.**	EC-528410
6.	EC-472250	**14.**	EC-528429
7.	IC-398065	**15.**	EC-390249
8.	IC-559388	**16.**	IC-202826

(70.72% polymorphism) and 45 bands were monomorphic (table-2). On average, the total number of bands generated per primer was 6.4, of which 4.6 were polymorphic and the 1.8 were monomorphic. Primer OPB-3, OPB-7 and OPC-5 were showed 100% polymorphism, while OPB-10 and OPB-17 were showed 85.71% polymorphism. The primer OPB-18, OPB-20 were showed 83.34 percent polymorphism and Primer OPC-18, OPC-19, OPO-11, OPO-15 were showed minimum percent of polymorphism (50%)

Table 2: RAPD primers with the number of amplified products

S. No.	Primer	Sequence	Total no of bands	Mono-. morphic bands	Polym-orphic bands	% polymo rphism
01.	OPB-1	GTTTCGCTCC	8	3	5	62.50
02.	OPB-3	CATCCCCCTG	7	0	7	100.00
03.	OPB-6	TGCTCTGCCC	4	1	3	75.00
04.	OPB-7	GGTGACGCAG	6	0	6	100.00
05.	OPB-9	TGGGGGACTC	5	2	3	60.00
06.	OPB-10	CTGCTGGGAC	7	1	6	85.71
07.	OPB-11	GTAGACCCGT	7	2	5	71.43
08.	OPB-12	CCTTGACGCA	7	2	5	71.43
09.	OPB-17	AGGGAACGAG	7	1	6	85.71
10.	OPB-18	CCACAGCAGT	6	1	5	83.34
11.	OPB-20	GGACCCTTAC	12	2	10	83.34
12.	OPC-1	TTCGAGCCAG	11	3	8	72.73
13.	OPC-2	GTGAGGCGTC	6	2	4	66.67
14.	OPC-5	GATGACCGCC	6	0	6	100.00
15.	OPC-7	GTCCCGACGA	5	2	3	60.00
16.	OPC-8	TGGACCGGTG	7	2	5	71.43
17.	OPC-9	TGGACCGGTG	4	1	3	75.00
18.	OPC-11	AAAGCTGCGG	5	2	3	60.00
19.	OPC-15	GACGGATCAG	6	2	4	66.67
20.	OPC-18	TGAGTGGGTG	4	2	2	50.00
21.	OPC-19	GTTGCCAGCC	6	3	3	50.00
22.	OPO-11	GACAGGAGGT	8	4	4	50.00
23.	OPO-15	TGGCGTCCTT	4	2	2	50.00
24.	OPO-16	TCGGCGGTTC	5	2	3	60.00
25.	OPO-20	ACACACGCTG	7	3	4	57.14
Total			160	45	115	-
Mean (Average)			6.4	1.8	4.6	70.72

compared to others. The number of bands ranged from 4 (OPB-6, OPC-9, OPC-18, OPO-15) to 12 (OPB-20) with an average of 6.4 bands/primer and 4.6 bands/primer were polymorphic. In RAPD analysis, the average of polymorphism percentage was 70.72 ranged from 50 to 100 (table-2). Minimum polymorphism percentage (50%) were recorded for primer OPC-18, OPC-19, OPO-11, OPO-15 and the maximum polymorphism percentage (100%) were recorded for primer OPB-3, OPB-7, OPC-5; followed by primer OPB-10 and OPB-17 (85.71% polymorphism). Out of 25 RAPD primers, only three oligonucleotide primers were generated polymorphic bands showing 100% polymorphism are shown in fig. 1. The RAPD profiles showed a high level of genetic variability among the genotypes of cowpea.

Prasanthi *et al.* (2012) reported total 120 RAPD fragments, of which 109 bands (90%) were polymorphic among 30 cowpea genotypes using 30 RAPD markers. Yadav *et al.* (2013) reported total of 77 bands in twelve isolates of *Bipolaris sorokiniana* of wheat using 20 RAPD primers, out of which 68 bands were polymorphic showing high range of variability (84.42% polymorphism). Bukhari *et al.* (2015) investigated 45 genotypes of common bean using 19 RAPD primers in which 253 total bands produced, 236 bands (94.22%) were polymorphic. Srujana and Lakshmi Bhavani (2016) studied 5 high yielding cultivars of cowpea by using 12 RAPD primers and detected 230 bands, 109 bands (91.1%) were found to be polymorphic. Shafiqul *et al.* (2017) examined genetic diversity in 13 ricebean varieties and their 11 narrow leaf crosses in which 147 total amplicons were scored, out of which 91 (61.9%) showed polymorphism indicating fair amount of variation at DNA levels and Percent polymorphism ranged from 38.9 percent to 59.8 percent. Thakur *et al.* (2018) studied genetic diversity among 54 genotypes of field and garden pea using 30 RAPD primers in which 168 amplicons were scored; of which 154 were polymorphic revealing 89.3% of polymorphism.

In this study, the average number of bands per RAPD primer was 6.4 compared with Khan *et al.* (2015) reported 6.67 bands per primer using 20 RAPD markers among 6 cowpea germplasm. But, in our present study the minimum and maximum number of polymorphic bands shows the polymorphism level on the basis

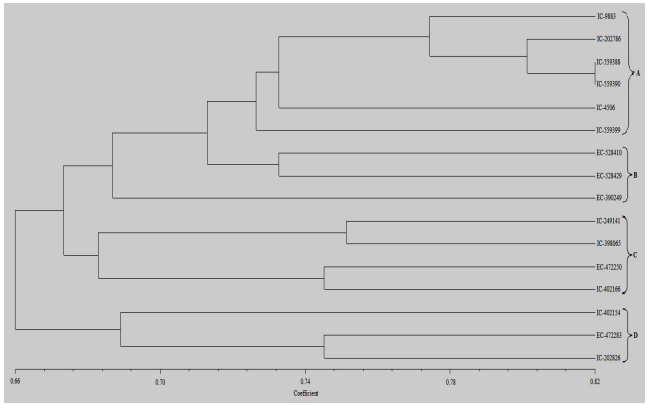

Fig. 1: UPGMA based cluster analysis of 16 cowpea using RAPD

of maximum and minimum amplification. Percent polymorphism values ranged from 50% to 100% with an average of 70.72% indicating a significant competency of RAPD markers for studying the polymorphism level available in the cowpea genotypes. These results proved RAPD markers to be good indicators of morphological divergence (Talebi *et al.*, 2008).

Cluster analysis

RAPD data were used to construct pairwise grouping of the cowpea genotypes by using software NTSYS-pc. The genetic similarity coefficient for 16 cowpea genotypes on the basis of 25 RAPD markers ranged from 0.544 to 0.825 (table-3). Maximum similarity coefficient value occurred between IC-559388 and IC-559390 was 82.5% and the minimum similarity coefficient value occurred between EC-472283 and EC-528410 was 54.4%. RAPD based fingerprinting, the genetic similarity value ranging from 0.544 to 0.825 which indicate the significant diversity (54% to 82%) among the genotypes used for this study. A dendrogram was constructed by clustering of 16 cowpea genotypes are shown in Fig. 1. The resulting dendrogram classified the 16 cowpea genotypes into 4 distinct clusters *i.e.* cluster A, B, C, and D comprising of 6, 3, 4 and 3 cowpea genotypes, respectively (table-4).

Cluster A grouped into two subcluster *viz.*; A1 and A2. Subcluster A1 consisted of 4 cowpea genotypes namely IC-9883, IC-202786, IC-559388 and IC-559390, which showed the maximum similarity coefficient occured between IC-559388 and IC-559390 with a value of 0.825 and the minimum similarity coefficient occured between IC-9883 and IC-202786 with a value of 0.763. Subcluster A2 consisted of only two cowpea genotypes namely IC-4506 and IC-559399, which showed the similarity coefficient value of 0.706. Cluster B consisted of 3 cowpea genotypes namely EC-528410, EC-528429 and EC-390249, which showed the maximum similarity coefficient occured between EC-528410 and EC-528429 with a value of 0.738 and the minimum similarity coefficient occured between EC-528410 and EC-390249 with a value of 0.688. Cluster C grouped into two subcluster *viz.*; C1 and C2. Subcluster C1 consisted of only two cowpea genotypes namely IC-249141 and IC-398065, which showed the similarity coefficient value of 0.756. Subcluster C2 consisted of only two cowpea genotypes namely EC-472250 and IC-402166, which showed the similarity coefficient value of 0.750. Cluster D consisted of 3 cowpea genotypes namely IC-402154, EC-472283 and IC-202826, which showed the maximum similarity coefficient occured between EC-472283 and IC-202826 with a value of 0.750 and the minimum

Table 3: Genetic similarity coefficient of 16 cowpea genotypes derived from RAPD marker

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1.000															
2	0.675	1.000														
3	0.756	0.681	1.000													
4	0.763	0.763	0.694	1.000												
5	0.706	0.706	0.650	0.731	1.000											
6	0.675	0.725	0.669	0.750	0.681	1.000										
7	0.700	0.650	0.756	0.663	0.644	0.750	1.000									
8	0.788	0.738	0.694	0.800	0.731	0.688	0.638	1.000								
9	0.700	0.688	0.656	0.663	0.631	0.750	0.675	0.613	1.000							
10	0.713	0.688	0.681	0.700	0.631	0.663	0.650	0.675	0.663	1.000						
11	0.713	0.625	0.669	0.588	0.694	0.663	0.713	0.613	0.663	0.700	1.000					
12	0.788	0.775	0.694	0.813	0.781	0.750	0.663	0.825	0.638	0.700	0.575	1.000				
13	0.656	0.694	0.663	0.719	0.725	0.719	0.744	0.719	0.581	0.669	0.544	0.794	1.000			
14	0.694	0.706	0.738	0.694	0.700	0.681	0.719	0.769	0.594	0.644	0.631	0.744	0.738	1.000		
15	0.619	0.706	0.650	0.694	0.650	0.644	0.669	0.731	0.681	0.656	0.631	0.719	0.688	0.725	1.000	
16	0.688	0.663	0.656	0.700	0.744	0.688	0.638	0.650	0.713	0.688	0.750	0.688	0.706	0.694	0.644	1.000

Table 4: Distribution of 16 cowpea genotypes into different clusters

S. No.	Cluster number	No. of cowpea genotypes	Genotypes
1.	A	04	IC-9883, IC-202786, IC-559388, IC-559390, IC-4506, IC-559399
2.	B	03	EC-528410, EC-528429, EC-390249
3.	C	02	IC-249141, IC-398065, EC-472250, IC-402166,
4.	D	02	IC-402154, EC-472283, IC-202826

similarity coefficient occured between IC-402154 and IC-202826 with a value of 0.688.

In recent years a number of studies have been undertaken to evaluate the genetic diversity and phylogenetic relationship in plant genetic resources. Prasanthi *et al.* (2012) evaluate 30 cowpea genotypes grouped into three groups at a similarity coefficient 25 and similarity index ranged from 0.463 to 0.784 on the basis of Jaccard's coefficient using UPGMA. Several workers have also been reported the utility of RAPD technique in studying the diversity of crop genotypes (Zannou *et al.*, 2008; Malviya and Yadav, 2010; Malaviya *et al.*, 2012; Motagi *et al.*, 2013; Patil *et al.*, 2013; Kole *et al.*, 2015). The use of appropriate statistical method especially in case of RAPD investigation is very important to create genetic variation more perfect. RAPD analysis has been developed to be a good applicant for the identification of crop variety. This method has been employed in present study during analysis of RAPD polymorphism. Results derived from this study would be highly useful in cowpea breeding programs and may be used for further crop improvement using advance marker systems. In conclusion, the results indicate the occurrence of moderate genetic variability along with the elite cowpea genotypes. RAPD markers are helpful in the evaluation of cowpea diversity and the selection of a core collection to improve the efficiency of genotype management for use in cowpea breeding and conservation.

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