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Genetic Diversity Analysis of Cowpea by RAPD Markers

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ABSTRACT: Random Amplified Polymorphic DNA (RAPD) assay was conducted for molecular genetic analysis of six cowpea (*Vigna unguiculata* L. Walp germplasms) i.e. BARI Cowpea, BARI Felon-1, Ranirhat, Ruma, BF06001 and BF060011, to observed genetic variability and relatedness among them. Out of 15 decamer random primers, three were used to amplify genomic DNA and the primers yielded a total of 20 RAPD markers of which 11 were considered as polymorphic. The maximum number of bands (9) (25%) produced by the primer S1184, whereas the least number of bands (5) (15%) was generated by the primer OPA10. The average scorable and polymorphic bands produced by the three primers were 6.67, and 3.67, respectively. The percentage of polymorphic loci was 55. The UPGMA dendrogram based on Nei's (1972) genetic distance between different pairs were correlated with their sources of origin. The dendrogram indicated segregation of six germplasms of cowpea into two main clusters; the main clusters are further divided into sub clusters. In sub cluster I, BARI Cowpwa-1, Ranirhat and Ruma formed sub sub-cluster I; BARI Felon-1 formed alone sub sub-cluster II. Again, sub sub-cluster I; Ruma formed alone cluster and BARI Cowpea-1 and Ranirhat formed sub sub-cluster III.

KEYWORDS: Genetic diversity, *Vigna unguiculata*, polymorphism, RAPD, marker.

1 INTRODUCTION

Cowpea [*Vigna unguiculata* (L). Walp] is a tropical grain legume widely distributed in sub-Saharan Africa, Asia, Central and South America as well as parts of southern Europe and the United States (Singh and Emechebe, 1997). Due to its high protein content (20-25%), cowpea plays a major role in human nutrition (Singh and Emechebe, 1997). It tolerates low fertility soil due to its high rate of nitrogen fixation (Eloward and Hall, 1987). The study of genetic diversity is important in a crop breeding program for the selection of suitably diverse parents to obtain heterotic hybrids as well as for germplasm characterization and conservation. Various morphological, biochemical and molecular markers are used for the characterization of germplasms. Molecular marker-based characterization is a useful complement to morphological and physiological characterization of cultivars, because they are plentiful, independent of tissue or environmental effects and allow cultivar identification in the early stages of plant development (Miller and Tanksley, 1990).

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Assessment of genetic diversity in cowpea genotypes would facilitate development of cultivars for specific production constraints by providing an index of parental lines to be used in breeding programmes. Keeping up the above points in mind, a research was conducted with the following objectives:

1. To reveal nuclear DNA level variation within each of the cowpea germplasms.
2. To determine the variation between the individuals among different germplasms.
3. To discriminate the different germplasms based on different levels of nuclear DNA variations.

2 MATERIALS AND METHODS

Six germplasms of Cowpea viz. BARI Cowpea-1, BARI Felon-1, Ranirhat, Ruma, BF06001 and BF060011 were used in the study. The varieties were different in their parent and also in breeding strategies. Seeds were collected from the BARI (Bangladesh Agricultural Research Institute).

Young and actively growing fresh leaf tissues were collected from a total of 18 individuals (three individuals per variety) for the isolation of genomic DNA. Total genomic DNA was isolated from cowpea leaves following Phenol: Chloroform: Isoamyl alcohol purification and ethanol precipitation method.

2.1 EXTRACTION OF GENOMIC DNA

The reagents and methods were used for the isolation of total genomic DNA. In brief, approximately 0.2-0.3 gm of leaf tissues were cut into small pieces, homogenized and digested in extraction buffer [50 mM Tris-HCl, pH 8.0, 25 mM EDTA (Ethylene diamine tetra acetic acid), 300 mM NaCl and 1% SDS (Sodium Dodecyl Sulphate) overnight at 35˚C. DNA was purified by successive extraction with Phenol: Chloroform: Isoamylalcohol (25 : 24 : 1, v/v/v). DNA was precipitated first using 0.6 volume of isopropanol, pelleted by centrifugation, then re-suspended in TE buffer (10mM Tris-HCl,1 mM EDTA, pH 8.0). DNA was re-precipitated by adding two volumes of absolute ethanol in the presence of 0.3 M sodium acetate, and pelleted by centrifugation. The pellets were then washed with 70% ethanol, air-dried and re-suspended in an appropriate volume of TE buffer. Finally, the DNA samples were stored at –20°C. DNA quality was checked by electrophoresis in a minigel and quantified using a spectrophotometer.

2.2 PRIMER SELECTION

Initially, fifteen primers of random sequence were screened on a sub-sample of two randomly chosen individuals from two different varieties to evaluate their suitability for amplifying cowpea RAPDs that could be scored accurately. Primers were evaluated based on intensity of bands, consistency within individual, presence of smearing, and potential for population discrimination. A final subset of three primers out of five exhibiting good quality banding patterns were selected for analysis of the whole sample set of the six germplasms of cowpea.

2.3 PCR AMPLIFICATION AND AGAROSE GEL ELECTROPHORESIS

The amplification conditions were based on Williams *et al.* (1990) with some modification. PCR reactions were performed on each DNA sample in a 10 µ1 reaction mix containing the following reagents: (For 20 reaction)

- 10x Ampli *Taq* polymerase buffer = 20 µl
- dNTPs = 20 µl
- Primers = 40 µl
- $MgCl_2$ = 12 µl
- Template DNA = 20 µl
- Ampli *Taq* DNA polymerase = 4 µl
- ddH_2O = 84 µl

During the experiment, PCR buffer, dNTPs, primer and DNA samples solutions were thawed from frozen stocks, mixed by vortexing and kept on ice. DNA template were pipetted first into PCR tubes compatible with the thermocycler used. A pre-mix was then prepared in the course of the following order: reaction buffer, dNTPs, DNA template and sterile distilled water. *Taq* polymerase enzyme was then added to the pre-mix. The pre-mix was then mixed up well and aliquoted into the tubes

that already contain primer. The tubes were then sealed and placed in a thermocycle and the cycling was started immediately.

DNA amplification was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf). The reaction mix was preheated at 94°C for 3 minutes followed 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 36°C and elongation or extension at 72°C for 2 minutes. After the last cycle, a final step of 7 minutes at 72°C was added to allow complete extension of all amplified fragments. After completion of cycling program, reactions were held at 4°C.

The amplified product from each sample was separated electrophoretically on 1.4% agarose gel contain ethidium bromide in 1X TBE buffer at 120 V for $1^1/_2$ hrs. A molecular weight marker DNA (20bp ladder) was eletrophoresed alongside the RAPD reactions. DNA bands were observed on UV-translluminator and photographed by a Gel Documentation System.

2.4 DATA ANALYSIS

RAPD markers were scored visually of their presence (1) or absence (0), separately for each germplasm of cowpea and each primer. For more accuracy, two independent persons performed band scoring. Bands not identified by the two readers were considered as non-scorable. The scores obtained using all primers in the RAPD analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, Nei's (1973) gene diversity, population differentiation (G_{ST}), gene flow (N_m), gene distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Mean) dendrogram among populations using POPGENE (version 1.31) (Yeh *et al*., 1999) computer program.

Estimation of gene frequencies of RAPD loci was based on the assumption of a two allele system. Of the two alleles, only one is capable of amplification of a RAPD band by primer annealing at an unknown genomic position (locus). The other is the 'null' allele incapable of amplification, mainly because of loss of the primer annealing site by mutation. The two allele assumption is in most cases acceptable, because codominant loci showing band shifts are few (Elo *et al*., 1997; Welsh and McClelland, 1990). In this system only a null homozygote is detectable as negative for the RAPD-band of interest. Under the assumption of Hardy-Weinberg equilibrium, the null allele frequency (q) may be (N/n)1/2, where N and n are the number of band negative individuals observed and the sample size, respectively. The frequency of the two allele (P) is 1-q. The assumption of the two allele system enables us to calculate the Nei's genetic distance (Nei's, 1972) from the RAPD pattern.

The similarity index values (SI) between the RAPD profile of any two individuals on the same gel were calculated from RAPD markers according to the following formula:

Similarity Index (SI) = $2N_{AB}/ (N_A + N_{B)}$

Where, N_{AB} is the number of RAPD bands shared by individual A and B and N_A and N_B, are the numbers of fragment scored for each individual respectively (Lynch, 1990). Within germplasm similarity [S_i] was calculated as the average of SI across all possible comparisons between individuals within a germplasm. Between germplasm similarity (S_{ij}) was calculated as the average similarity between randomly paired individuals from germplasm I and j (Lynch, 1991).

3 RESULTS AND DISCUSSION

Six Cowpea germplasms were analyzed and a total of 20 bands were scored. The selected three primers produced comparatively higher number of high intensity bands with minimal smearing. These three primers produced a total of 20 RAPD markers of which 11(55%) were considered as polymorphic (Table 1).

The banding patterns of different germplasms using primers S1184, OPA10 and OPP13 were showed in figure. 1

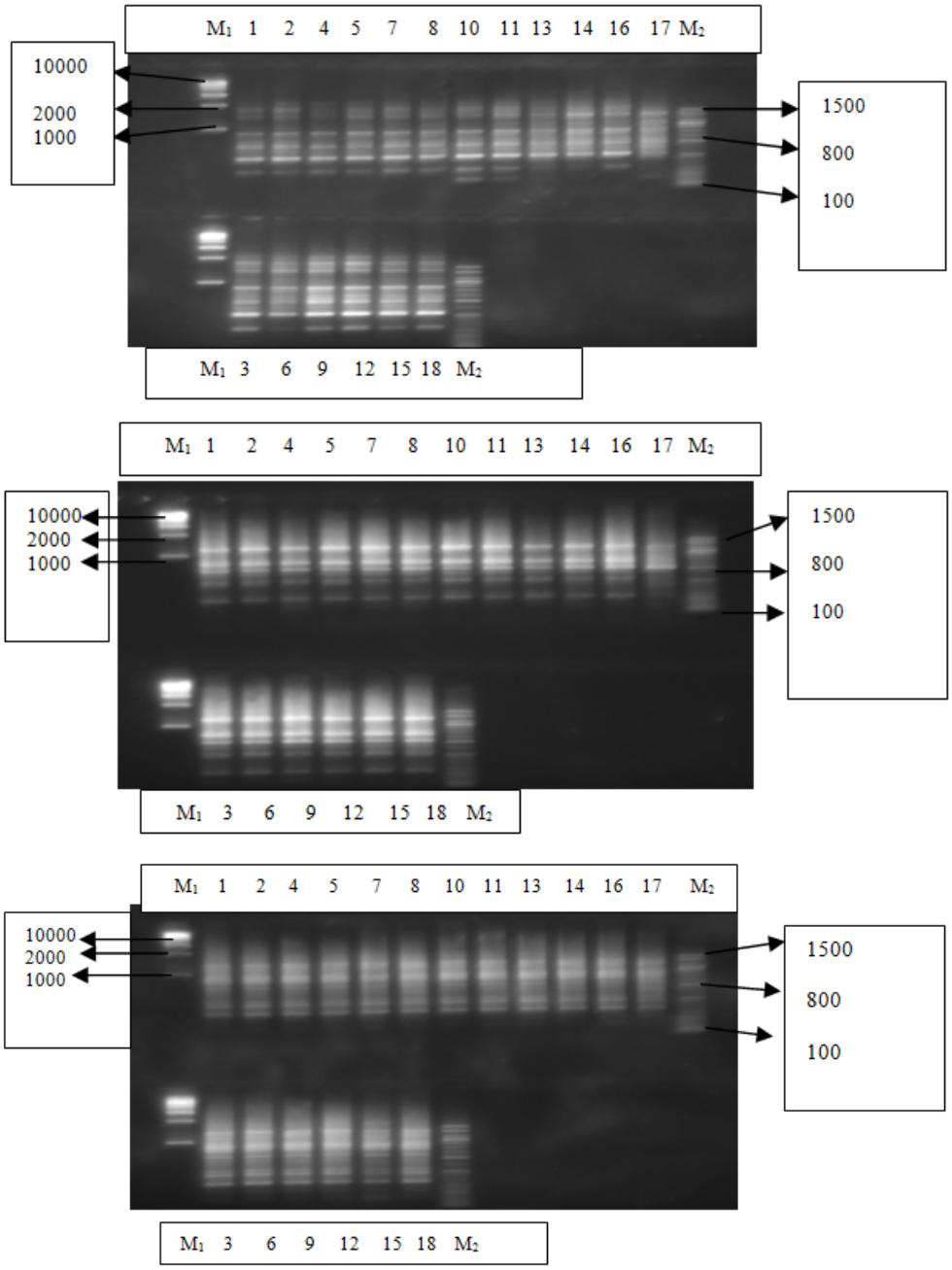

Fig. 1. RAPD profile of six different germplasms of cowpea using S1184, OPA10 and OPP13 primer Lane 1-3: BARI Cowpea-1; 4-6: BARI Felon-1; 7-9: Ranirhat; 10-12: Ruma; 13-15: BF06001; 16-18: BF060011. M_1: 1kb DNA ladder, M_2: 20bp DNA ladder

The maximum number of bands (9) produced by the primer S1184, whereas the least number (5) generated by primer OPA10. On the other hand, the primer S1184 amplified higher percentage of polymorphic bands (25%), while the primer OPA10 generated the least (15%) polymorphic bands. The average scorable and polymorphic bands produced by the three primers were 6.67, and 3.67, respectively. Weak bands result from low homology between the primer and the pairing site on the DNA strand (Thormann *et al*., 1994).

Table 1. RAPD primers with corresponding bands scored together with polymorphic bands observed in six cowpea germplasms

Primer codes	Sequences (5'-3')	G+C (%)	Total number of bands scored	Number of polymorphic bands
S1184	GACGGCTATC	60	9	5
OPA10	GTGATCGCAG	60	5	3
OPP13	GGAGTGCCTC	70	6	3
Total			20	11
Average			6.67	3.67

3.1 INTRA AND INTER-GERMPLASM SIMILARITY INDICES

The intra-germplasm similarity indices (Si) were found higher (ranged from 76.90-100% with an average of 93.53%) than the inter germplasm similarity indices (Sij) (ranging from 87.22-99.26% with an average of 93.30%). For the population of cowpea, intra-germplasm similarity indices were found higher in BARI Cowpea-1 and Ranirhat germplasm than the other four cowpea germplasms. On the other hand, individuals BF060011 germplasm showed the lowest similarity indices.

Inter-germplasm means of the pair-wise similarity indices (Sij) ranged from 87.22 to 99.26%. The highest similarity index of 99.26% was found between BARI Cowpea-1 Vs Ranirhat, while Ruma Vs BF060011 showed the least inter-germplasm similarity index (87.22%) (Table 7B). Band sharing based intra-germplasm similarity indices were higher than inter-germplasm similarity indices. Among the three primers, OPP13 showed highest intra-germlpasm (95.52%) and inter-germplasm similarity indices (96.16%), while S1184 generated lowest intra-germplasm similarity indices (89.83%) and inter-germplasm similarity indices (88.51%).

3.2 POLYMORPHIC LOCI AND GENE DIVERSITY

The highest proportion polymorphic loci (45%) were also found in the germplasm BF060011, whereas no polymorphism found for BARI Cowpea-1 (Table 2). However, the highest and the lowest Nei's gene diversity values were found in BF060011 and BARI Cowpea-1.

Table 2. Number and proportion of polymorphic bands, gene diversity obtained in different six cowpea germplasms

Germplasm	No. of polymorphic loci	Proportion of polymorphic loci (%)	Gene Diversity
BARI Cowpea-1	0	0.00	0.000
BARI Felon-1	2	10.00	0.0488
Ranirhat	1	5.00	0.0150
Ruma	2	10.00	0.0394
BF06001	4	20.00	0.0788
BF060011	9	45.00	0.2102

3.3 GENE FLOW

The pair-wise gene flow (Nm) values were presented in Table 3. The highest gene flow (Nm) value was found in Ruma Vs BF060011 germplasms pair which was 0.1681 while the lowest gene flow value (0.0001) was observed in BARI Cowpea-1 Vs Ranirhat germplasms pair (Table 3).

Table 3. Gene flow value (Nm) in different varietals pair (below diagonal)

Germplasm	BARI Cowpea-1	BARI Felon-1	Ranirhat	Ruma	BF06001
BARI Felon-1	0.0293				
Ranirhat	0.0001	0.0300			
Ruma	0.0066	0.0378	0.0035		
BF06001	0.0915	0.0736	0.0841	0.0936	
BF060011	0.1612	0.1190	0.1541	0.1681	0.1116

3.4 NEI'S (1972) GENETIC IDENTITY AND GENETIC DISTANCE

The highest Nei's (1972) genetic distance (0.1996) was observed in Ruma Vs BF060011 germplasms pair whereas, the lowest genetic distance (0.0017) was estimated in BARI Cowpea-1 Vs Ranirhat germplasms pair (Table 4). Furthermore, high level of genetic distance was found in BARI Cowpea-1 Vs BF060011 (0.1886); Ranirhat Vs BF060011 (0.1830); BARI Felon-1 Vs BF060011 (0.1515) germplasms pair and low level of genetic distance was observed in Ranirhat Vs Ruma (0.0091); BARI Cowpea-1 Vs Ruma (0.0107) germplasms pair.

Table 4. Summary of Nei's genetic distance values between six cowpea germplasms

Germplasm	BARI Cowpea-1	BARI Felon-1	Ranirhat	Ruma	BF06001	BF060011
BARI Cowpea-1						
BARI Felon-1	0.0345					
Ranirhat	0.0017	0.0367				
Ruma	0.0107	0.0471	0.0091			
BF06001	0.1001	0.0874	0.0942	0.1064		
BF060011	0.1886	0.1515	0.1830	0.1996	0.1475	

3.5 DENDROGRAM

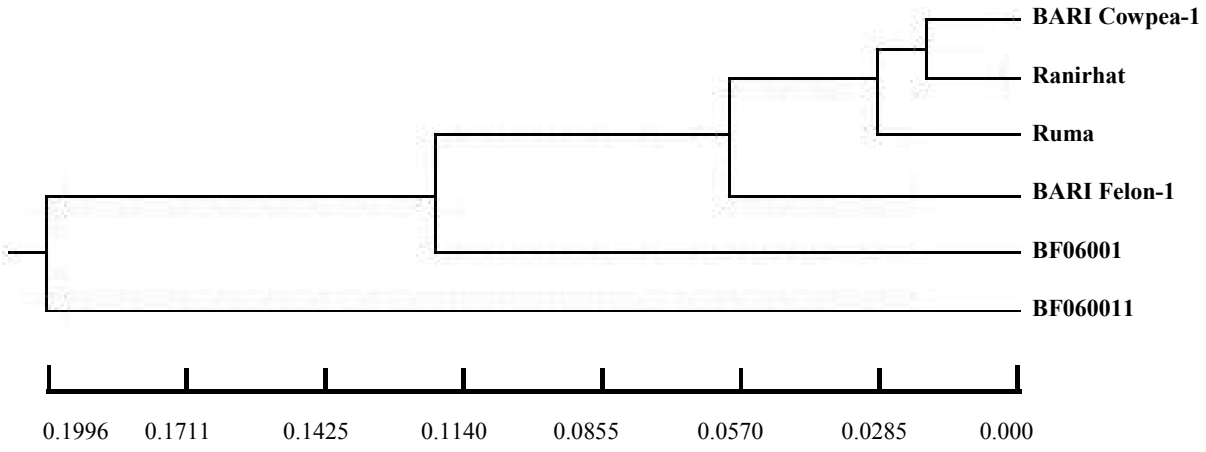

Fig. 2. UPGMA dendrogram based on Nei's genetic distance, summarizing the data on differentiation between six cowpea germplasms according to RAPD analysis

A dendrogram based on Nei's (1972) genetic distance using UPGMA, indicates segregation of six germplasms of cowpea into two main clusters: BARI Cowpea-1, BARI Felon-1, Ranirhat, Ruma, BF06001 grouped in cluster I while BF060011 in cluster II (Fig. 2). In cluster I, BARI Cowpea-1, BARI Felon-1, Ranirhat and Ruma formed sub cluster I; BF06001 formed alone sub cluster II. In sub cluster I, BARI Cowpwa-1, Ranirhat and Ruma formed sub sub-cluster I; BARI Felon-1 formed alone sub sub-cluster II. Again, sub sub-cluster I; Ruma formed alone cluster and BARI Cowpea-1 and Ranirhat formed sub sub-cluster III.

The results indicate that the low or high level of genetic distance exists between germplasms with their same or different origins. Ruma Vs BF060011 germplasms pair showed highest genetic distance (0.1996), as they released from different source. On the other hand, BARI Cowpea-1 Vs Ranirhat germplasms pair showed lowest genetic distance (0.0017) as they were released from same source.

In this study, the RAPD technique was found to discriminate six cowpea germplasms. Germplasms showing higher intra-population similarity and lower proportion of polymorphic loci are likely to have less heterozygosity as compared to those showing less intra-germplasm similarity and higher proportion of polymorphic loci. In other words, germplasms having higher similarity are more homogenous groups. According to the results, BF060011 showed more genetically diversified comparative to the other germplasms.

The present study showed that out of 20 RAPD markers, 11 scorable bands (55%) were considered as polymorphic. This proportion of polymorphism is higher compared to some previous RAPD analysis in cowpea e.g. 46.5% of cowpea landraces were polymorphic in Pandey *et al*. (2004), 26.27% of cowpea landraces in Zannou *et al.* (2008) and lower polymorphic from 64.5% of cowpea landraces in Sharawy and Fiky, (2003). This difference can be attributed to the primers used and the genotypes evaluated.

Genetic variation refers to the differences in the heredity constitutions of the individuals of a species and it is important in maintaining the developmental stability and biological potential of plant species. This study indicates the highest genetic variation between Ruma Vs BF060011 and lowest genetic variation between BARI Cowpea-1 Vs Ranirhat that can be used for breeding programmes that aim to improve cowpea varieties. The results also revealed that the genetic base among these cowpea varieties is rather narrow.

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