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Genetic diversity analysis in cowpea (*Vigna unguiculata* (L.) Walp.) Genotypes for storage seed protein profile

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Abstract
An investigation was carried out on 58 genotypes (accessions and mutants of cowpea variety RC 101 and RC 19) of cowpea for seed storage protein profile using Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to study diversity among the genotypes. The storage proteins were extracted from cotyledons of the seeds to represent both water and salt soluble proteins. Gels revealed distinct bands within the molecular weight (MW) range of 80 to 14 kD. The binary data was generated by scoring the presence and absence of every protein bands and used to compute Jaccard's similarity coefficient with the help of NTSYS-pc programme. The similarity coefficient matrix was used to construct a dendrogram of the 58 cowpea genotypes by the un-weighted paired group method using arithmetic averages (UPGMA).

Keywords: Genetic Diversity, SDS – PAGE, Dendrogram, Cowpea

1. Introduction
Cowpea [*Vigna unguiculata* (L.) Walp] is an annual, self-pollinated, leguminous crop**.** Cowpea is cultivated around the world not only as a pulse but also as a vegetable crop. It has many common names including Black-eyed pea, Crowder pea, Southern pea and lobia. Cultivated cowpea varieties are adapted to hot and dry condition. Because of its high protein content (20-25%), cowpea has been referred to as "poor man's meat". In agriculture cowpea is considered as one of the oldest legumes used as protein source for people and livestock (Steele, 1972). The crop is also known to provide quick and thick cover on ground and thus helps in conservation of soil. It is grown as an alternative crop in dry land farming. It provides protein rich diet to human and nutritious fodder to livestock. Young leaves, pods and peas of cowpea contain vitamins and minerals which have fuelled its usage, both for human consumption and animal feeding as well. *In situ* decomposition of root residues of cowpea crop contributes organic matter and associated nutrients to the soil. There are several diverse uses of cowpea due to which the varietal requirement in terms of plant type, seed type, maturity, pattern of use and growth are diverse from region to region. Therefore cowpea breeding programme becomes more complex and no single variety can be suitable for all the objectives (Barrett, 1987) [2]. Thus, there is need to develop varieties suitable for a specific region and /or use. However, production is constrained by low and variable grain yield, grain quality, susceptibility to diseases and pests and the absence of improved cultivars. Therefore genetic diversity in cowpea must be characterized and exploited for development of genotypes with improved yield potential. For an effective breeding programme the characterization of genetic diversity for making choice of parents for hybridization is important. seed storage protein profile, on one hand, is an important consideration to be taken in account when drawing inferences from genetic diversity studies based only on morphological traits, such a protein profile, on the other hand, directly refers to its nutritional status. The research work related to genotype characterization in cowpea using SDS-PAGE of storage seed protein is very scanty.

Material and methods
The present investigation was conducted on 58 genotypes of cowpea (*Vigna unguiculata*) including stable mutants of cowpea variety RC-101 and RC-19 available at Department of Plant Breeding and Genetics, S.K.N. College of Agricuture, Jobner the 58 genotypes were grown in kharif season and the seed produced were used to estimate seed protein content in

the present study. The list of genotypes along with their source of procurement and their appearance are presented in Table. 1. SDS-PAGE was conducted according to procedure of Laemmli (1970) [6] with minor modification described by Tripathy *et al.* (2010) [15], Sharma (2012) and Choudhary (2013) [3]. For protein extraction, seed coat and embryo were removed and cotyledons were ground and sieved to get a fine powder. Proteins were extracted by grinding, first, in 1ml of doubled distilled water followed by subsequent grinding in 1ml of 1M NaCl, respectively as described by Sharma (2012). Extracted protein samples (1ml) were transferred into Eppendorf tubes(1.5ml tube) and centrifuged for 3 minutes at 10,000 rpm.One half milliliter of (0.5) supernatant was transferred into a fresh Eppendorf tube and denatured with 0.5ml cracking buffer by keeping at 80^0 C in a water bath for 15 minutes. Bromophenol blue (BPB) added to the cracking buffer served as tracking dye to monitor the movement of protein bands in the gel. These samples were loaded into the wells of the polyacrylamide gel slab prepared for electrophoresis. The steps followed for gel preparation. The electrophoresis was carried out on BioRAD vertical gel electrophoresis equipment (Model: Protein II Xi Cell) along with its cooling unit with a power supply maintained at 30 mA for four and half hours. Two separate gels were run under similar electrophoretic conditions in order to check the reproducibility of the results. After electrophoresis gels were stained with Coomassie brilliant blue R 250 followed by distaining and finally washing in tap water. The washed gels were put in clear transparent bags for further use in photography. The molecular weights of the dissociated polypeptides were determined by using standard molecular weight marker. The thoroughly distained gels were put in clear transparent polythene bags for further use in photography. Gels were scored for the presence (1) and absence (0) of every protein subunit band. These binary data were used to analyze using NTYSYS –pc (Numerical Taxonomy System, Version 2.1 (Rohlf, 2000) software. The SIMQUAL sub- programme was used to calculate the Jaccard's coefficient using following formula (Jaccard, 1908) [5].

Jaccard's coefficient= $N_{AB}/ (N_{AB}+N_A+N_B)$

Where, N_A and N_B represent number of bands in sample A and sample B, respectively. N_{AB} is the number of bands shared in the samples. Similarity matrices as computed by the programme were used to construct the UPGMA (Sneath and Sokal, 1973) [12]. Dendrogram was constructed to elucidate the diversity among the accessions studied. Statistical stability of the branches in the cluster was estimated by bootstrap analysis with 1000 replicates, using Winboot software program (Yap and Nelson, 1996) [16]. Mobility of protein in SDS gel electrophoresis was expressed as relative mobility (Rf) with respect to the tracking dye, bromophenol blue. Rf value of protein markers of known size was used to generate a standard curve by plotting the molecular weights against the Rf value on a semi-log graph. The molecular weight of the unknown protein can beextrapolated from its Rf value using the standard curve asdepicted in Appendix III prepared as per the procedure described inthe Bio-Rad bulletin.

Table 1: List of cowpea genotypes used in the study and their source of collection

S. No.	Cowpea genotype	Source
1.	GC-501	IVT-1 CAZRI trial 2010
2.	GC-703	IVT-1 CAZRI trial 2010
3.	NBC-2	IVT-1 CAZRI trial 2010
4.	CPD-118	IVT-1 CAZRI trial 2010
5.	NBC-1	IVT-1 CAZRI trial 2010
6.	NBC-3	IVT-1 CAZRI trial 2010
7.	SUBHARA	IVT-1 CAZRI trial 2010
8.	GOA LOCAL	IVT-1 CAZRI trial 2010
9.	PGCP-12	IVT-1 CAZRI trial 2010
10.	CPD-119	IVT-1 CAZRI trial 2011
11	JOB-11(Mutant of RC-101)	IVT-1 CAZRI trial 2011
12.	JOB-80BR (Mutant of RC-101)	IVT-1 CAZRI trial 2011
13.	KBC-4	IVT-1 CAZRI trial 2011
14.	CPD-121	IVT-1 CAZRI trial 2011
15.	GC-817	IVT-1 CAZRI trial 2011
16.	KBC-5	IVT-1 CAZRI trial 2011
17.	PTB-1	IVT-1 CAZRI trial 2011
18.	CPD-83	IVT-1 CAZRI trial 2011
19.	GC-815	IVT-1 CAZRI trial 2011
20.	GC-810	IVT-1 CAZRI trial 2011
21.	DC-15	IVT-1 CAZRI trial 2011
22.	HC-38	IVT-1 CAZRI trial 2011
23.	CPD-103	IVT-State trial Durgapura 2011
24.	CPD-132	IVT-State trial Durgapura 2011
25.	CPD-134	IVT-State trial Durgapura 2011
26.	CPD-127	IVT-State trial Durgapura 2011
27.	CPD-142	IVT-State trial Durgapura 2011
28.	CPD-136	IVT-State trial Durgapura 2011
29.	CPD-78	IVT-State trial Durgapura 2011
30.	RC-19	Check variety
31.	CPD-129	IVT-State trial Durgapura 2011
32.	CPD-77	IVT-State trial Durgapura 2011
33.	MUTANT-1(Mutant of RC-19)	Dept. Cowpea Research, SKNCOA
34.	MUTANT-2(Mutant of RC-101)	Dept. Cowpea Research, SKNCOA
35.	MUTANT-3(Mutant of RC-101)	Dept. Cowpea Research, SKNCOA

36.	MUTANT-4(Mutant of RC-101)	Dept. Cowpea Research, SKNCOA
37.	MUTANT-5(Mutant of RC-101)	Dept. Cowpea Research, SKNCOA
38.	MUTANT-6(Mutant of RC-101)	Dept. Cowpea Research, SKNCOA
39.	MUTANT-7(Mutant of RC-101)	Dept. Cowpea Research, SKNCOA
40.	MUTANT-8(Mutant of RC-19)	Dept. Cowpea Research, SKNCOA
41.	MUTANT-9(Mutant of RC-101)	Dept. Cowpea Research, SKNCOA
42.	MUTANT-10(Mutant of RC-19)	Dept. Cowpea Research, SKNCOA
43.	MUTANT-11(Mutant of RC-19)	Dept. Cowpea Research, SKNCOA
44.	MUTANT-12(Mutant of RC-101)	Dept. Cowpea Research, SKNCOA
45.	CPD-108	IVT-1 CAZRI trial 2009
46.	GC-525	IVT-1 CAZRI trial 2009
47.	GC-521	IVT-1 CAZRI trial 2009
48.	PGCP-6	IVT-1 CAZRI trial 2009
49.	JOB-129(Mutant of RC-101)	IVT-1 CAZRI trial 2009
50.	CP-107	IVT-1 CAZRI trial 2009
51.	CPD-105	IVT-1 CAZRI trial 2009
52.	PHULE-CP-5040	IVT-1 CAZRI trial 2009
53.	PHULE-CP-5030	IVT-1 CAZRI trial 2009
54.	RC-101	Check variety
55.	GC-3	Check variety
56.	CPD-115	IVT-1 CAZRI trial 2010
57.	GC-723	IVT-1 CAZRI trial 2010
58.	DCS-47-1	IVT-1 CAZRI trial 2010

Results and discussion

Characterization of genotypes based on seed storage proteins/subunits is well documented in different crops including legumes; blackgram (Ghafoor and Ahmad, 2005) [4], *Capsicum annuum* L. (Anu and Peter, 2003) [1], *Vigna* spp (Rao *etal*., 1992) [9]; *Vignaunguiculata* (Sharma 2012 and Choudhary 2013) [3], fenugreek (Pareek, 2014) [8], and mustard (Parashar, 2014) [7].

Legume seeds, in general, predominantly contain albumins (20-35%) and globulins (43-55 %). Of these, albumins are water soluble and globulins are salt soluble. These two proteins together account for 63-90% of the total seed proteins (Tchiagam *et al*., 2011) [14]. Therefore, in the present investigation only albumins and globulins were analyzed.

Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS–PAGE) as described by Laemmeli (1970) [6] is a powerful and dependable technique for characterization of proteins. In principle, it involves extraction of proteins from the target materials, e.g. cotyledons, leaves etc, followed by its denaturation into polypeptides in the presence of beta-mercaptoethenol (that breaks disulphide bonds) and SDS. SDS has two functions: it denatures secondary, tertiary and quaternary structures by binding to hydrophobic protein regions and then its binding confers a net negative charge on the resulting denatured proteins or the polypeptide. The protein/subunits are then separated through a gel (polyacrylamide) in an electric field according to their masses. In the present investigation, the proteins from the cotyledons were isolated as described by Tripathy *et al*. (2010) [15]. Since albumins are water soluble and globulins are salt soluble, therefore, their simultaneous extraction was done as per modified method of Sharma (2012) and Choudhary (2013) [3]. The protein extracts from the cotyledons of 58 genotypes (i.e. accessions and mutants) were prepared as described above and the gel plates were loaded with 14 samples at a time along with marker protein in the first lane (shown in figure. 1). The comb used in these experiments could develop 15 wells for loading of the samples. The marker protein has invariably shown 8 distinct protein bands between 66 to 20 kD MW. A comparison of banding pattern revealed that there are five distinct regions of protein. The first region corresponded to 80 kD molecular weight(MW).The second region is relatively darkly stained and thick with two sub units of approximately 56 kD MW. The third region revealed three sub units around 43 kD MW. The fourth region was comprised of 3 lightly stained bands between 35 kD-20 kD and last region seemed to be unresolved (near to the 14 KD MW). Sharma (2012) and Choudhary (2013) [3] have also observed same number of regions of protein subunit bands in their study on 52 cowpea accessions and 38 mutants of cowpea. Win *et al*. (2011) [17] have also described a similar picture of electrophoregram in cowpea accessions of Myanmar and have identified 5 regions on the basis of banding pattern within the similar molecular weight range of 97kD to 15kD.

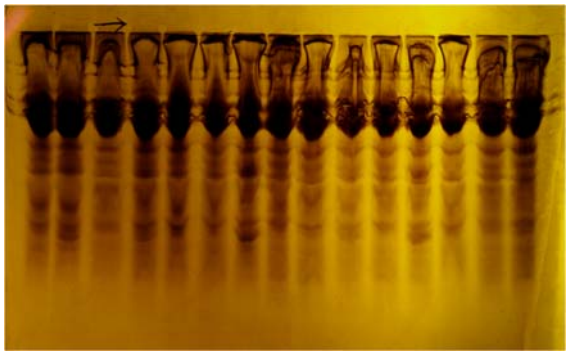

Fig 1: A close up view of electrophoregram of cowpea genotypes

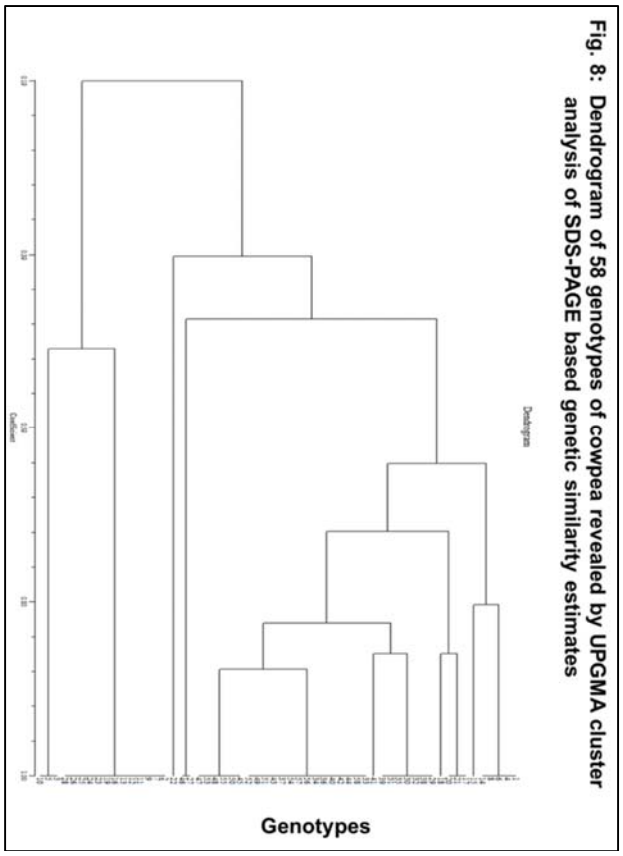

Fig. 8: Dendrogram of 58 genotypes of cowpea revealed by UPGMA cluster analysis of SDS-PAGE based genetic similarity estimates

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