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GENETIC DISSIMILARITIES IN COWPEA (*VIGNA UNGUICULATA* (L.) WALP.) FOR PROTEIN PEPTIDES AND THEIR SIGNIFICANCE FOR QUANTITATIVE TRAITS LOCI

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Abstract

Cowpea *(Vigna unguiculata* (L.) Walp.), germplasm comprising of diverse 138 accessions was evaluated for 23 physiological and agrononic characters during summer 2000 at NARC, Islamabad. The same material was analyzed for total seed protein using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Out of 40 protein subunits, 31 were polymorphic and 9 were monomorphic. Out of polymorphic bands, 23 were observed significant for various quantitative trait loci. SDS-PAGE provided a tool for germplasm discrimination based on genetic differences in seed storage protein comparison in cowpea. The factors affecting quantitative traits may occur as individual genes or gene clusters scattered throughout the genome, therefore, quantitative traits were expected differently at several loci. Variation in 17 quantitative traits (root weight, branches, chlorophyll contents, pods plant^{-1}, leaf area trifoliate^{-1}, plant height, root length, biological yield^{-1}, seeds pod^{-1}, number of locules^{-1}, seed set percentage, grain yield^{-1}, harvest index, 100-seed weight, seed length, seed width and pod width) was significantly associated with various protein sub-units, however, the actual number of QTLs could be fewer because several of these traits were correlated. Variation at protein peptides in the vicinity of QTLs may be an indication of genetic variation potentially available to breeding programmes for improving yield potential. Expansion of genetic base for cowpea breeding might be accomplished by systematic use of germplasm that differ from common banding pattern and are known to be associated with variation in quantitative traits.

Introduction

Cowpea is an important tropical crop, indigenous to Africa from where it was introduced to other tropical and sub-tropical countries (Cobley & Steele, 1976). The nutritional value of cowpea lies in its protein contents of 20-50%. It is a cheap source of quality protein, phosphorus, iron and vitamins and therefore, an excellent substitute of meat, eggs and other protein sources (Carangal *et al.*, 1979). Annual worldwide production of cowpea is estimated as 2.5 millions tons of dry bean harvested from 9 millions hectares. About 20% of the total grown cowpea is consumed as fresh vegetable. It is cultivated on marginal soils in Pakistan, especially in NWFP and Northern Punjab, on an area of about 16.9 thousands hectares with an annual production of 7.8 thousand metric tones (Bashir, 1992). It is planted as spring (March to June) or summer (July to October) crops.

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Presently, biochemical markers have been used to reveal the phylogenetic relationships and genetic diversity in crop plants (Yasui *et al.*, 1985; Rao *et al.*, 1992; Fatokum *et al.*, 1993; Vaillancourt *et al.*, 1993; Zink *et al.*, 1994). Among biochemical techniques, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is widely used due to its validity and simplicity for describing genetic structure of crop germplasm (Moller & Spoor, 1993; Rabbani, *et al.*, 2001; Ghafoor *et al.*, 2002).

From the perspective of genetic analysis, biological data fall into two broad categories; 1) quantitative traits (many agronomical features) with continuous variation governed by several to many genes; and 2) qualitative data (morphological or molecular markers) with discrete phenotypes governed by one to several genes. Importantly, these two types of traits may similarly be variants of a single genetic theme, distinguishable only by the magnitude of allelic substitution effects (Comstock, 1978; Robertson, 1989). Cowpea is the least researched crop in the country and no reports on biochemical analyses are available. Therefore present study was conducted to investigate the extent of genetic diversity on the basis of SDS-PAGE markers and their association with various quantitative traits loci (QTLs) for future utilization in markers assisted breeding.

Materials and Methods

One hundred and thirty eight diverse accessions of cowpea were planted in an augmented design under field conditions at the National Agricultural Research Centre (NARC) Islamabad, during summer 2000. This center is situated 33^0 44' N latitude and 73^0 08' E longitude at an altitude of 540 masl. Germplasm was provided by the genebank of Plant Genetic Resources Programme, which has been collected from different parts of the country. Eighty four accessions were collected from various parts of the country that represents a wide ecogeographic variation from dry mountainous to irrigated plains and sandy arid region of Pakistan. The exotic germplasm was obtained from International Institute of Tropical Agriculture (IITA), Nigeria (49 genotypes), University of Riverside California, USA (4 genotypes) and China (1 genotype). One check (local) was included in this study after every 10 rows. One row of 4 m lengths was planted for each accession with 75 cm row spacing, whereas plant spacing was kept at 20 cm. Recommended cultural practices were followed throughout the crop season to get healthy crop (Anon., 2001). Plant and agronomic characters were recorded following IPGRI descriptor for cowpea (IBPGR, 1983). For agronomic characters, 10 plants of each accession were sampled at random for data collection.

Protein extraction

For the extraction of proteins, single seed was ground to fine powder in a mortar and pestle. Sample buffer (400 µl) was added to 0.01 g of seed flour as extraction liquid and mixed thoroughly in eppendorf tube with a small glass rod. The extraction buffer contained (0.5 M Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol and 5% 2-mercaptoethanol in final concentrations. Bromophenol Blue (BPB) was added to the sample buffer as tracking dye to watch the movement of protein in the gel. To purify extraction, the homogenate samples were mixed thoroughly by vortexing and centrifuged at 15,000 rpm for 5 minutes at RT. The extracted crude proteins were recovered as clear supernatant, transferred into new 1.5 ml eppendorf tubes and stored at –20 °C until electrophoresis.

Electrophoresis

Seed proteins were analyzed through slab type SDS-PAGE using 11.25% polyacrylamide gel. Electrophoresis was carried out at 100 V for 3 hours until the Bromo Phenol Blue (BPB) marker reached the bottom of the gel. In order to check the reproducibility of the method, two separate gels were run under similar electrophoretic conditions. The molecular weights of the dissociated polypeptides were determined by using molecular weight protein standards "MW-SDS-70 kit" from Sigma Chemical Company, USA. SDS-PAGE of total protein was carried out in polyacrylamide slab gels in the discontinuous buffer system according to the method of Laemmli (1970). The separating gels contained 11.25% of Acrylamide 0.135% by weight of N-N-muthylene-bis-acrylamide in 1M Tris-HCl buffer, pH 8.8, with 0.27 % SDS. The gels were polymerised chemically by the addition of 20 µl by volume of tetramethylethylene-diamine (TEMED) and 10% Ammonium Persulfate (APS). The stacking gels consisted of 30% Acrylamide and 0.8% N.N-methylene-bis-acrylamide in 0.25% M Tris-HCl buffer, pH 6.8, containing 0.2% SDS. The stacking gels were polymerised chemically in the same way as for the separation gel. The electrode buffer contained Tris-glycine (9.0g Tris HCl and 43.2 g glycine per 3 liters buffer solution at a pH 8.9) with 3.0 g (0.1%) SDS. Protein supernatant 8 µl were applied into the wells in staking gel sample wells with a microsyringe.

Staining and destaining

After electrophoresis, the gels were stained with 0.2% (W/V) Coomassie Brilliant Blue R250 dissolved in a solution containing 10% (V/V) acetic acid, 40% (V/V) methanol and water in the ratio of 10:40:60 (V/V) for one hour. Gels were then destained by washing with a solution containing 5% (V/V) acetic acid, 20% (V/V) methanol and water in the ratio of 5:20:75 (V/V) until the color of background disappeared and electrophoresis bands were cleanly visible. After destaining, the gels were dried using gel drying processor for about 100 minutes.

Data analysis

Depending upon presence and absence of polypeptide bands, similarity index was calculated for all possible pairs of protein types. To avoid taxonomic weighing, the intensity of bands was not taken into consideration, only the presence of bands was taken as indicative. Presence and absence of bands were entered in a binary data matrix. Based on results of electrophoretic band spectra, similarity index was calculated for all possible pairs of protein types electrophorograms (Sneath & Sokal, 1973). On the basis of genetic dissimilarities, a dendrogram was constructed to investigate genetic clusters with the help of computer software "STATISTICA" for Windows 2000. The quantitative and molecular data were analyzed for comparisons of means for quantitative traits with SDS-PAGE markers. Genes affecting the variation of QTL were determined by dividing quantitative data into two groups on the basis of presence or absence of protein peptide. The group means of quantitative characters were calculated and regression analysis was applied to compute the probabilities that two group means were equal, using computer software "SPSS" and "MS EXCEL".

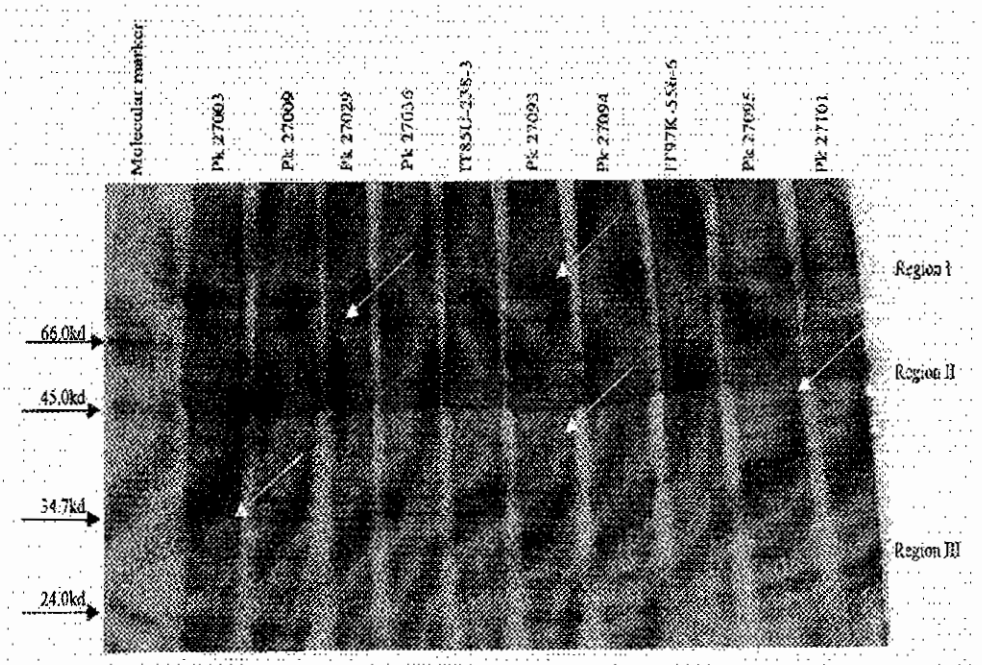

Fig. 1. Intra-specific variation in proteins peptides of *Vigna unguiculata* (L.) Walp. The molecular marker used in the gel was SDS-70 Kit. The arrows indicate variation in different regions.

Results and Discussion

Genetic diversity

The SDS-PAGE conducted in various combinations revealed that 11.25% acrylamide gel concentration, 8 µl of sample gave the best resolution. SDS-PAGE markers exhibited diversity in cowpea with variation in protein bands ranging from 24.0 to 66.0 kd (Fig. 1). In total, 40 protein bands were recorded ranging from the molecular weight of 24 to 66 kd. Many protein subunits of lower MW were also observed but due to inconsistency in reproducibility they were not recorded. Occasionally, variation was also observed in the density or sharpness of a few bands but this variation was not taken in consideration. The gel was divided into three regions and all the three regions exhibited variation with major differences in the region II. Moller & Spoor (1993) suggested 5 regions in *Lolium* spp., and observed major differences in the regions B, C and D. In the present studies, intra-specific variation was limited among cowpea accessions and no differences were observed for major bands.

The dendrogram based on SDS-PAGE markers revealed 11 clusters (Fig. 2). Cluster I comprised of two accessions, one (Cowpea Narowal) from Pakistan and one (UCR-9704) from USA. One accession (27167) that was collected from Punjab was in cluster II (Bhatti *et al.*, 1997). Cluster III comprised of I accession that was obtained from IITA, Nigeria. Cluster IV consisted of 5 genotypes, all from exotic origin i.e., 4 from IITA, and one from USA. Cluster V comprised of 20 accessions, 13 from IITA, 3 from NWFP province and 4 from Punjab. Cluster VI had 8 genotypes all from local origin, 4 from

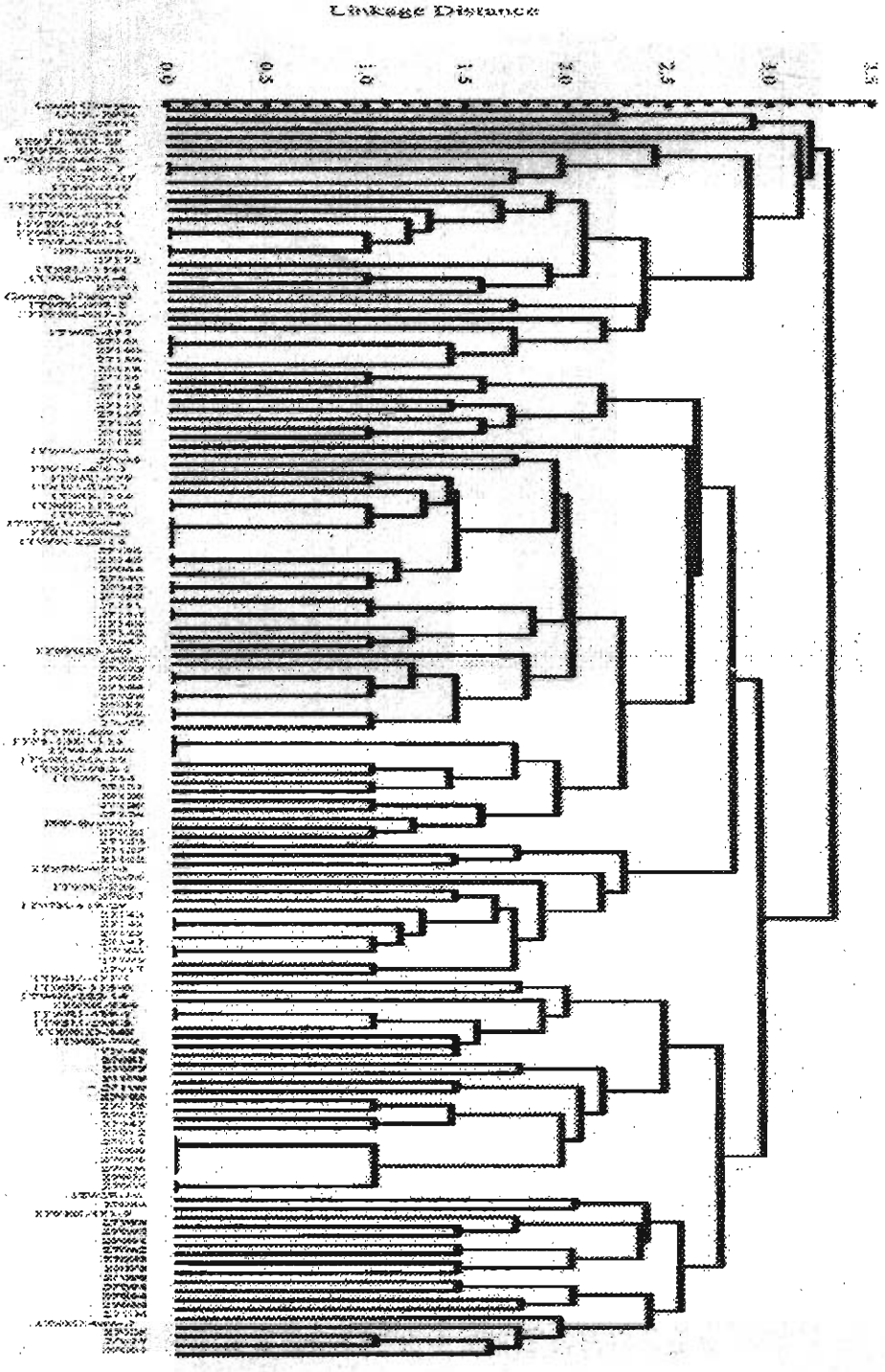

Fig. 2. Cluster diagram of 138 accessions of cowpea based on SDS-PAGE markers

Table 1. Significant means detected by regression for quantitative traits grouped for presence or absence of SDS-PAGE markers in *Vigna unguiculata* germplasm

Bands	CC	LA	DF1	PH	BR	PD	RL	RW	PL	SP	LP	SSP	SW	SL	SWD	BY	GY	HI
B1							2.33			2.28	2.20	1.90				2.51	3.40	3.26
B2a				2.77										2.77				
B2b													2.04					
B3a	2.09							2.10										
B4a		2.11							2.11									
B5														2.63	2.46			
B6							2.70							2.88			2.71	
B6a							1.77										1.77	2.66
B7a			2.25					2.22	2.25	2.84	2.53	3.04						
B8						2.27	2.70								1.77	2.28	2.70	
B8a				3.80				2.46						3.81				
B8b				2.07						2.6	2.00	2.92		2.07				
B9		2.13							2.13					1.96				
B9b				2.35		2.07								2.35			2.07	
B9c	2.60							2.61						2.12				
B10	2.84				2.41			2.84						2.32		2.14		
B10a						2.97											2.97	
B11														3.59			1.91	
B12					2.18										2.34			
B13										2.08				2.61	3.07			
B14	2.13	2.54						2.13	2.54									
15	3.22			2.82				3.23						2.82				
B16				2.47										2.47				
Total	5	3	1	6	2	3	4	6	3	4	3	3	1	13	4	3	7	2

CC- chlorophyll contents, LA- leaf area trifoliate^{-1} DF1- days to Ist flower, PH- plant height 9, BR -number of branches^{-1}, PD- pods plant^{-1}, RL- root length, RW- root weight, PL- pod length, SP- seeds pod^{-1}, LP- locules pod^{-1}, SSP- seed set percentage, SW- 100-seed weight, SL- seed length, SWD- seed width, BY- biological yield, GY- grain yield and HI- harvest index

Table 2. Protein markers associated with quantitative traits in *Vigna unguiculata*.

Bands	Association with QTLs
B1	Root length, seeds pod^{-1}, locules pod^{-1}, seed set percentage, biological yield, grain yield, harvest index
B1b	No Association
B2a	Plant height^{1}, seed length
B2b	100-seed weight
B2c	Chlorophyll contents, root weight, seeds pod^{-1}, seed set percentage, grain yield, harvest index
B3a	No association
B3b	No association
B4a	Leaf area trifoliate^{-1}, pods plant^{-1}
B5	Seed length, seed width
B6	Root length, seed length, grain yield
B6a	Root length, seed length, grain yield
B6b	No association
B7a	Days to Ist flower, root weight, pod length, seeds pod^{-1}, locules pod^{-1}, seed set percentage
B8	Pods plant^{-1}, root length, seed width, biological yield, grain yield
B8a	Plant height, root weight, seed length
B8b	Plant height, seeds pod^{-1}, number of locules pod^{-1}, seed set percentage, seed length
B9	Leaf area trifoliate^{-1}, pod length, seed length
B9a	No association
B9b	Plant height, pods plant^{-1}, seed length, grain yield
B9c	Chlorophyll contents, root weight, seed length
B10	Chlorophyll contents, number of branches^{-1}, root weight, seed length, biological yield
B10a	Pods plant^{-1}, grain yield
B11	Seed length, grain yield
B12	Number of branches^{-1}, seed width
B13	Seeds pod^{-1}, seed length, seed width
B14	Chlorophyll contents, leaf area trifoliate^{-1}, root weight, pod length
B14a	No association
B14b	No association
B15	Chlorophyll contents, plant height, root weight, seed length
B15a	No association
B16	Plant height, seed length

NWFP and 4 from Punjab. Cluster VII consisted of 44 accessions, 9 from NWFP, 17 from Punjab, 16 from IITA, and 2 were of exotic origin. Cluster VIII consisted of 14 accessions which were from different origins; 4 from NWFP, 1 from Gilgit, 1 from Islamabad, 5 from Punjab and 3 from IITA. Cluster IX comprised of 24 lines, 9 from *IITA and 15 were collected from Punjab. Cluster X consisted of 9 accessions, 2 from* IITA, 6 from Punjab and one from NWFP. Cluster XI consisted of 9 accessions which originated from China (27104), Baluchistan (27094), Punjab (27099, 27089, 27025, 27018 ,27001), NWFP (27098) and IITA (IT85F-867-5).

Significance of SDS-PAGE markers for QTLs

Besides genetic variation, screening analysis for marker bands to detect QTLs were carried out and some significance of protein peptides were observed in determining QTLs in cowpea. Out of 40 protein bands, 31 were polymorphic and others were monomorphic. Twenty three bands were associated with various QTLs on the basis of regression. Out of 920 combinations (QTLs X total markers), 74 combinations were significantly associated with various QTLs (Table 1). The association of QTL with easily identifiable gene markers could permit a rapid and precise transfer of QTL into superior crop cultivars (Tanksley *et al.*, 1989; Tahir & Muehlbauer, 1995). Based on regression, 8 protein markers (B1, B2c, B6, B6a, B8, B8b, B9a, B10a, and B11) were observed significant for detection of yield and yield components (Table 2). Eleven protein bands (B2a, B2c, B3a, B4a, B8a, B8b, B9b, B10, B19a, B14, B15) were significantly associated with vegetative characters, whereas 8 (B1, B2b, B2c, B5, B7a, B8b, B9b, B13) were observed significant for detecting QTLs related to seed and pod characters. Seed protein electrophoreses have been successfully used to resolve the taxonomic and evolutionary problems along with their relation with QTLs in several crop plants (Ladizinsky & Hymowitz, 1979; Khan, 1992; Das & Mukarjee, 1995; Ghafoor *et al.*, 2003). It is a promising tool for distinguishing cultivars of a particular crop species (Gardiner & Forde, 1988; Jha & Ohri, 1996; Ghafoor *et al.*, 2002). Variation for SDS-PAGE was not observed for major bands rather weak bands were recorded with varying degrees of dissimilarities that indicated the conservative nature of the genome in *Vigna unguiculata.*

The factors or loci affecting variation in quantitative traits may occur as individual genes or gene cluster scattered throughout the genome, therefore, same quantitative traits may be expressed differently at several loci (Tahir & Muehlbauer, 1995). The use of molecular markers to locate genes controlling quantitative traits has been considered important in the analysis of such traits (Stuber *et al.*, 1982; Stuber, 1992; Kahler & Wehrhahan, 1986; Kjaer *et al.*, 1991; Mansur *et al.*, 1993). Variation in 18 traits out of 23 was significantly associated with 23 protein peptides; however, the actual number of QTL might be fewer because several of these traits were correlated. The association of QTL with easily identifiable markers could permit the rapid and precise identification and transfer of QTL into superior crop cultivars (Tanksley, 1983). The amount of information provided by this marker-based approach will depend on the type and number of markers, and their linkage relationship (Singh *et al.*, 1991). The frequency of these markers based of protein peptides for QTL are not very commonly observed since these protein subunits would tend to be simply inherited, whereas agriculturally important traits are usually polygenic in nature. The initial results are encouraging for locating factors that influence the expression of quantitative traits. However, the conclusions are specific to the sample investigated, and the environment in which the measurable traits were recorded.

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