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DIVERSITY OF AGRONOMIC TRAITS AND TOTAL SEED PROTEIN IN BLACK GRAM *VIGNA MUNGO* (L.) HEPPER

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This study evaluated agronomic traits of 111 genotypes of black gram *Vigna mungo* (L.) Hepper, mainly from Pakistan, to determine the extent of genetic diversity. Seed proteins were analyzed by SDS-PAGE. High genetic variance was observed for days to flowering, days to maturity, number of branches/plant, number of pods/plant, biomass/plant, grain yield/plant and harvest index, whereas low genetic variance was observed for pod length, seeds/pod and 100-seed weight in both years studied. SDS-PAGE of seed proteins showed low inter-accession diversity and no clear differentiation on the basis of origin or source. The genotypes in one cluster with similar agronomic characters did not necessarily belong to the same origin or source. The lack of a geographic pattern of germplasm collected from Pakistan was probably due to exchange of germplasm between neighbouring regions, or because germplasm represented progenies of the same ancestors. Clustering of advanced breeding lines along with an approved variety in one group revealed that only a portion of the genetic diversity has been exploited for improvement of black gram. It is suggested that the genetic base of cultivated black gram should be broadened by involving diverse parents in the breeding programme. Expansion of the genetic base for black gram breeding might be accomplished by systematic use of germplasm that differs in protein profiles and has better quantitative traits.

Key words: Black gram, *Vigna mungo* (L.) Hepper, cluster analysis, gel electrophoresis, genetic diversity, SDS-PAGE.

INTRODUCTION

Knowledge of genetic diversity is a useful tool in genebank management and in planning experiments, as it facilitates efficient sampling and utilization of germplasm by identifying and/or eliminating duplicates in the gene stock, and helps in the establishment of core collections. One practical application of knowledge of genetic diversity is in the design of populations for genome mapping experiments (Kaga et al., 1996). Characterization of germplasm using biochemical techniques (fingerprinting) has received attention because of the increased recognition of germplasm resources in crop improvement and in selection of desirable genotypes to be used in breeding programmes. Genetic markers are useful for screening germplasm with the minimum cost in time and labour (Nakajima, 1994). Seed protein patterns obtained by electrophoresis have been successfully used to resolve the taxonomic and evolutionary relationships among crops and their wild relatives (Rao et al., 1992; Das and Mukarjee, 1995). They can also be used for distinguishing cultivars of particular crop species (Jha and Ohri, 1996). However, a few studies indicated that cultivars could not be identified by the SDS-PAGE method, as the electrophoretic patterns of proteins were similar between cultivars (Ahmad and Slinkard, 1992; de Vries, 1996).

Among the biochemical techniques, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is widely used due to its simplicity and effectiveness for describing the genetic structure of crop germplasm. Seed storage proteins have been used as genetic markers in four major areas: (1) analysis of genetic diversity within and between accessions, (2) plant domestication in relation to genetic resource conservation and breeding, (3) establishing genome relationships, and (4) as a tool in crop improvement. SDS-PAGE is considered to be a practical and reliable method because seed storage proteins are largely independent of environmental fluctuations (Gepts, 1989, 1990; Murphy et al., 1990; Javaid et al., 2004; Iqbal et al., 2005).

Researchers can use information on genetic similarity to make decisions regarding selection of superior genotypes for improvement or for use as parents for the development of future cultivars through hybridization. This type of study has not yet been carried out for black gram *Vigna mungo* (L.) Hepper, although it is an

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important summer pulse crop of many South Asian countries including Pakistan, India, Nepal, Bangladesh, Thailand and Korea. In South Asian countries it is cultivated in a wide range of agro-ecological zones, particularly under rainfed conditions. The present study was initiated to study genetic diversity on the basis of agronomic traits and SDS-PAGE markers in black gram.

MATERIALS AND METHODS

A set of 111 genotypes was evaluated for agronomic traits during the summer seasons (mid July to end October) of 2000 and 2001. Of these, 75 accessions were collected from various parts of Pakistan (Fig. 1). These accessions were collected from all over the country: 33 from Punjab, 14 from North West Frontier Province (NWFP), 9 from Baluchistan, 10 from the Northern Areas (NA) and 9 from Azad Jammu and Kashmir (AJK) during the last two decades. Thirty advanced breeding lines (numbers starting with 90, 92 or 93 represent advanced breeding lines) were obtained from national breeders working on genetic improvement of black gram. One genotype (PL–2) was obtained from the Asian Vegetable Research and Development Centre (AVRDC), one (41016) from India, and three (43/5, RC-13, RC-19) from Afghanistan. Prior to experimentation, genotypes were self-pollinated for two years to establish homozygosity. Two 4-meter rows for each genotype were planted, with 10 cm intra-row and 75 cm inter-row spacing. The approved variety Mash 1 was repeated as the control after every 10 rows. Pesticides were sprayed to protect the crop from pests, especially white fly (*Bemisia tabaci* Genn.), a vector for Mungbean Yellow Mosaic Virus (MYMV). Data were recorded following IPGRI descriptors for *Vigna mungo* and *V. radiata* (IBPGR, 1985). Days to flowering and to maturity were recorded at 50% flowering and 90% maturity, and these variables were represented by a single value for each row. Plant height, branches/plant, pods/plant, grain yield (g) and biomass yield (g) were recorded for 10 plants sampled randomly. Pod length (cm) and seeds per pod were recorded for ten pods sampled at random within each genotype. The 100-seed weight (g) was recorded and the harvest index was determined as economic yield expressed as a percentage of total biomass.

SDS-PAGE ANALYSIS

For extraction of proteins, a single seed was ground to fine powder with mortar and pestle. Sample buffer (400 µl) was added to 0.01 g seed powder as extraction liquid and mixed thoroughly in an Eppendorf tube with a small glass rod. The extraction buffer contained the following final concentrations: 0.5 M Tris-HCl (pH 6.8), 2.5% SDS, 10% glycerol and 5% 2-mercaptoethanol. Bromophenol Blue (BPB) was added to the sample buffer as tracking dye to monitor the movement of protein in the gel. Seed protein was analyzed through slab-type SDS-PAGE using 11.25% polyacrylamide gel measuring 7 × 9 cm. Two separate gels were run under similar electrophoretic conditions in order to check the reproducibility of the method. The molecular weights of the dissociated polypeptides were determined using molecular weight protein standards (MW-SDS–70 kit, Sigma, U.S.A.). SDS-PAGE of total seed protein was carried out in a discontinuous buffer system according to the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue (CBB) and then destained till the background became transparent (Masood et al., 2003).

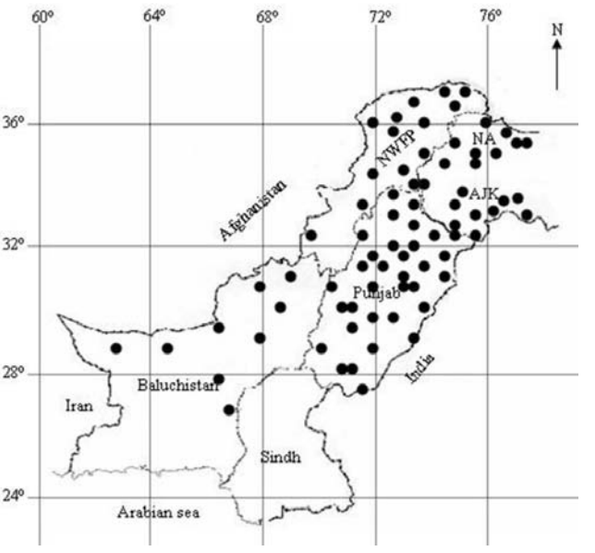

Fig. 1. Black gram germplasm collection sites (●) in Pakistan. NWFP – North Western Frontier Province; AJK – Azad Jammu and Kashmir; NA – Northern Areas of Pakistan.

DATA ANALYSIS

The data recorded were analyzed for simple statistics: means, standard deviations and heritabilities. Heritability was estimated as the ratio between genotypic and phenotypic variance (Singh and Chaudhry, 1985). The data were also analyzed by numerical taxonomic techniques using cluster and principal component analyses (Sneath and Sokal, 1973) with the Statistica and SPSS for Windows packages (SPSS, 1996; Statsoft, 2001). The means of each variable were standardized prior to cluster analysis to avoid effects due to differences in scale. After SDS-PAGE, staining and destaining, similarity indexes were calculated for all possible pairs of

TABLE 1. Basic statistics for quantitative characters in 111 accessions of black gram

Characters	2000				2001				*r*
	Mean±SE	σ	h^2	Check variety	Mean±SE	σ	h^2	Check variety	
Days to flowering	43±0.88	9.32	0.72	40±0.40	40±1.02	8.42	0.72	38±0.31	0.62*
Days to maturity	80±1.38	14.57	0.69	74±0.18	76±1.52	14.95	0.68	71±0.21	0.71*
Plant height	39.2±2.41	15.46	0.71	36.1±0.71	36.9±1.91	16.91	0.69	34.2±0.61	0.81*
Branches/plant	18.9±0.90	9.45	0.51	30.6±0.93	15.1±0.82	10.34	0.46	32.2±1.05	0.58*
Pods/plant	53±3.05	32.21	0.59	112.6±1.05	45.0±4.25	29.15	0.61	96.7±1.23	0.74*
Pod length (cm)	4.4±0.028	0.290	0.35	4.6±0.01	4.3±0.10	0.23	0.40	4.6±0.02	0.82*
Seeds/pod	6.2±0.058	0.609	0.41	6.4±0.04	6.3±0.02	0.51	0.49	6.4±0.03	0.80*
100-seed weight (g)	4.62±0.056	0.589	0.29	5.5±0.04	4.71±0.07	0.52	0.21	4.46±0.05	0.91*
Biological yield (g)	49.57±2.79	29.35	0.68	75.76±0.76	51.75±3.75	21.25	0.71	67.18±0.99	0.72*
Grain yield (g)	12.55±0.83	8.78	0.52	30.71±0.27	10.71±1.23	6.61	0.58	26.82±0.84	0.68*
Harvest index (%)	25.37±0.79	8.34	0.51	38.74±0.45	24.98±0.84	9.12	0.49	39.45±1.13	0.51*

σ – standard deviation; h^2 – heritability; *r* – correlation between 2000 and 2001 for various traits; *significant at $p < 0.01$.

protein types, based upon the presence or absence of polypeptide bands. To avoid taxonomic weighting, the intensity of bands was not taken into consideration; only the presence of the bands was taken as indicative. The presence and absence of bands were entered in a binary data matrix. Based on the results of electrophoretic band spectra, similarity indexes were calculated for all possible pairs of protein type electrophoretograms. The similarity matrix thus generated was converted to a dissimilarity matrix and used to construct a dendrogram by the unweighted pair group average method.

RESULTS

The results presented in Table 1 reveal high genetic variance and repeatability for all the traits except pod length, seeds/pod and 100-seed weight, which limited the scope of improvement of these traits through simple selection from the germplasm used in the present study. The high association between the two years for all characters indicated the consistency of experimental conditions.

AGRONOMIC TRAITS

Germplasm was classified into 3 clusters at 50 percent linkage distance. Cluster I consisted of 49 accessions, all of them of local origin (Fig. 2). Of these, 29 were advanced breeding lines along with the approved variety (Mash 1). Cluster II comprised 31 accessions. All the exotic germplasm was grouped in this cluster along with local accessions, and none of the advanced breeding lines were grouped in this cluster. All 3 accessions obtained from Afghanistan were grouped together, with minimum diversity between them, and they were closely related with 41016, an Indian genotype. Cluster III consisted of 31 accessions, all of which were of local origin. Of the 9 accessions collected from Baluchistan, 7 were grouped in this cluster. The accessions in this cluster were grouped together on the basis of high yield ability. Hence the accessions in this cluster are suggested for evaluation under a wide range of environmental conditions, to exploit the yield potential of black gram. On the basis of principal component analysis, the first two factors were plotted and are presented in Figure 3. The genotypes were scattered throughout the graph. Most of the genotypes were mixed, but genotypes originating from Afghanistan and India were separated from other accessions.

The mean values and standard deviations for each cluster on the basis of 2-year average performance are presented in Table 2. The accessions of cluster I were early-maturing but low-yielding, whereas the accessions grouped in cluster II were late-maturing and gave low to medium yield. Cluster III, comprised of 31 accessions all collected from Pakistan, gave high average yield and were medium to late in maturing. The genotypes from various sources differed considerably, and all the exotic genotypes were grouped in cluster II. This means that a low-level relationship between origin and clustering pattern could be established on the basis of cluster analysis.

SDS-PAGE ANALYSIS

The seed protein profiles of the majority of genotypes were similar, and only 46 exhibited differences in protein banding patterns from SDS-PAGE. In total, 15 protein bands were recorded. Protein subunits with lower MW (< 24 kD) were not considered due to lack of reproducibility. There was occasional variation in the density or sharpness of a few bands, but this variation

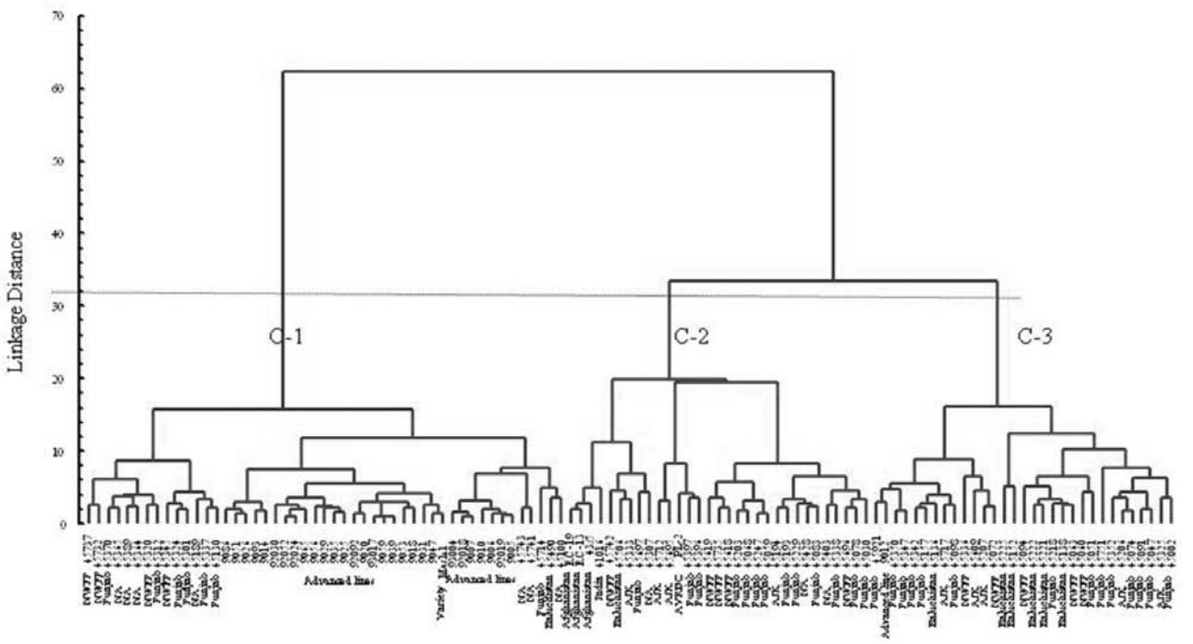

Fig. 2. Phenogram of 111 genotypes based on quantitative traits in black gram.

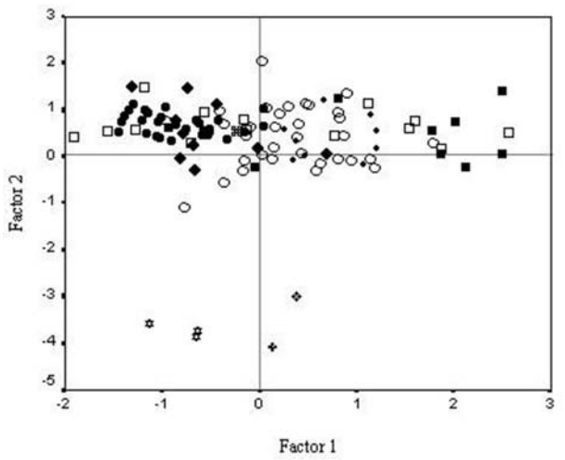

Fig. 3. Scatter diagram of 111 genotypes based on quantitative traits. ❍ – Punjab; ■ – Baluchistan; ❑ – NWFP; ◆ – Northern Areas; ❖ – AVRDC; ♦ – Azad Jammu and Kashmir; ⌘ – varieties; ● – advanced lines; ✠ – India; ✡ – Afganistan.

was not considered. Of 15 protein subunits, 9 were polymorphic and 6 were monomorphic (Fig. 4). Only polymorphic bands were included in principal component analysis (PCA) and dendrogram construction. On the basis of banding pattern, the gel was divided into four regions, and the first three regions of bands with MW > 24 kD were considered for polymorphism.

TABLE 2. Means and standard deviations for clusters (average of 2 years) based on agronomic characters

Character	Cluster I	Cluster II	Cluster III
Frequency	49	31	31
Days to flowering	37.00±5.81	49.00±7.53	46.00±10.54
Days to maturity	70.00±8.94	93.00±10.53	87.00±13.34
Plant height	48.70±6.79	40.70±9.61	31.90±4.26
Branches/ plant	17.50±8.54	16.40±6.62	23.90±11.56
Pods/branch	2.70±1.41	2.70±1.52	4.40±1.84
Pod length (cm)	4.30±0.20	4.50±0.36	4.50±0.30
Seeds/pod	5.90±0.34	6.40±0.85	6.50±0.50
100-seed weight (g)	4.45±0.55	4.74±0.64	4.81±0.53
Biomass/plant (g)	31.00±15.63	45.61±21.03	85.10±21.82
Grain yield (g)	7.44±3.91	9.71±5.76	24.08±6.40
Harvest index (%)	25.31±8.35	21.65±7.50	29.19±7.62

On the basis of SDS-PAGE, 14 clusters at 50% linkage distance were found (Fig. 5). The genotypes 45714, 45136, 45059, 45157, 45030 and 45002 did not show resemblance with any other, and all these were of local origin. Clusters I, VII, IX and X consisted of 2 accessions each, all locally collected. Cluster XI consisted of 23 accessions, one of which was obtained from

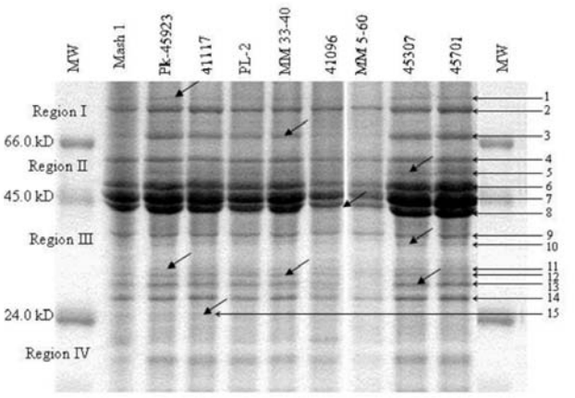

Fig. 4. Variation in seed proteins of *Vigna mungo* (L.) Hepper. Arrows represent the presence of a polymorphic band.

Afghanistan, 7 were advanced breeding lines, and the others locally collected germplasm. Cluster XII comprised a mixture of 58 accessions originating from various sources. The clustering pattern made on the basis of SDS-PAGE grouped the accessions differently and gave no clear indication of agronomic performance or origin/source. Similarly, in the graphic presentation of PCA, the genotypes overlapped because 65 genotypes did not exhibit polymorphism for SDS-PAGE markers (Fig. 6). For 46 genotypes, a major group closer to its origin was formed, and only a few genotypes were separated from the major group. The results of PCA and cluster analysis were in agreement for the material used in this study.

DISCUSSION

Evaluation of crop germplasm is essential to ensure its efficient and effective use. In the present investigation, high genetic variation was observed for all the characters except pod length, seeds/pod and 100-seed weight. This indicated that improvement through simple selection for traits with high genetic variance is possible, but for seed characters (pod length, seeds/pod, 100-seed weight), more germplasm from diverse sources should be incorporated to broaden the genetic base (Laghetti et al., 1998; Ghafoor et al., 2001). Though cluster analysis grouped genotypes with greater similarity for agronomic performance, it did not necessarily include all the genotypes from the same source or origin. Amur-

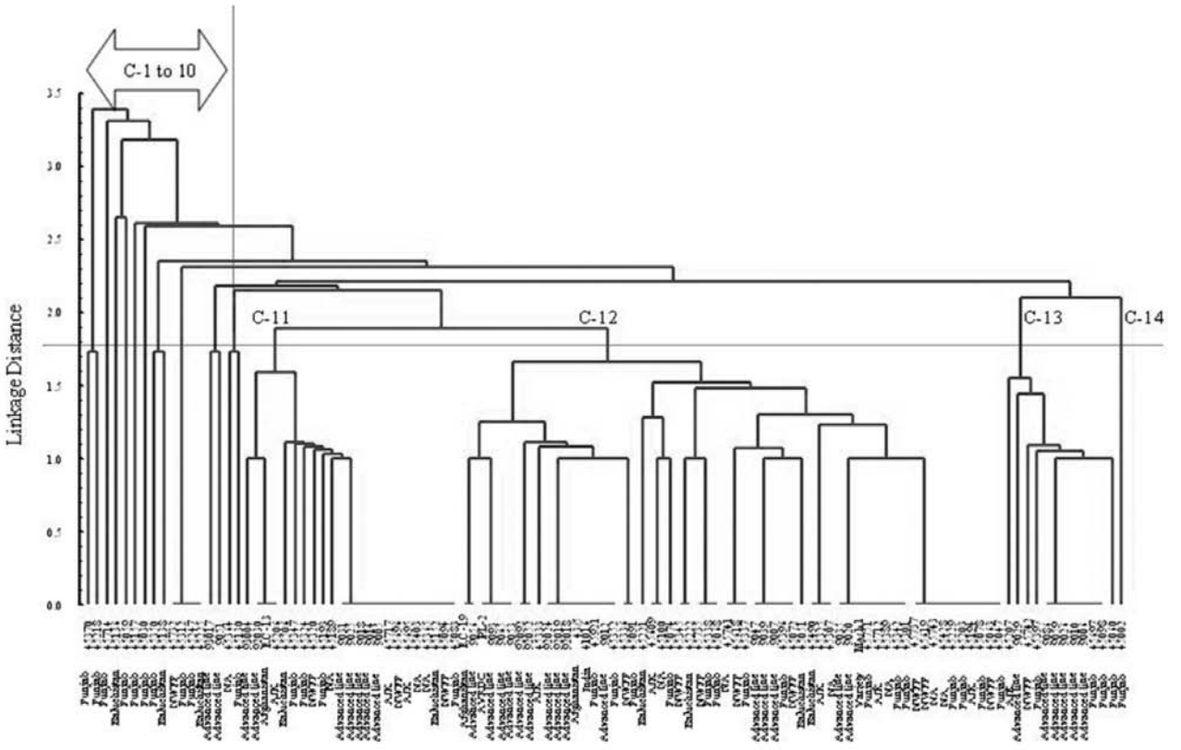

Fig. 5. Dendrogram of 111 genotypes based on SDS-PAGE markers in black gram.

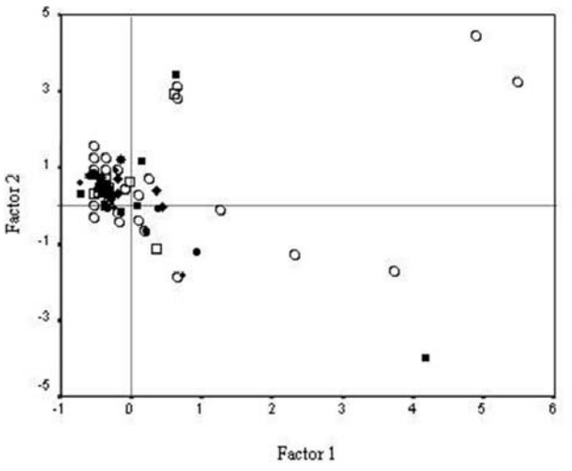

Fig. 6. Scatter diagram of 111 genotypes based on SDS-PAGE markers. ❍ – Punjab; ■ – Baluchistan; ❑ – NWFP; ◆ – Northern Areas; ❖ – AVRDC; ♦ – Azad Jammu and Kashmir; ⌘ – varieties; ● – advanced lines; ✠ – India; ✡ – Afganistan.

rio et al. (1993, 1995) and Ghafoor (2000) also reported a lack of correlation between agronomic performance and geographic origin. The indifferent clustering patterns of germplasm collected from Pakistan might be due to frequent exchange of germplasm between neighbouring regions, perhaps with the same ancestors. Cluster analyses based on agronomic traits was more reliable than SDS-PAGE analysis of protein peptides, where one group consisting of genotypes from various sources was observed. Only a few genotypes were scattered and separated from the main group. Of these, 8 were collected from Punjab, 2 from NWFP, 2 from Baluchistan and one from AJK, and one was an advanced breeding line. Based on agronomic traits, the grouping of advanced breeding lines along with an approved variety in one group revealed that only a portion of the genetic diversity of black gram has been exploited. The genetic base of cultivated black gram should be broadened, involving diverse parents in the breeding programme. The exotic germplasm, which exhibited some degree of separation on the basis of quantitative characters, was mixed together in one major cluster on the basis of SDS-PAGE analysis; it was not possible to identify accessions on the basis of source, and no relationship of markers with yield potential was observed.

Although SDS-PAGE showed diversity in the storage protein banding patterns of the present material, its magnitude was low. It needs to be broadened through collections and acquisition of germplasm from centres of origin. Variation on the basis of protein peptides has been reported by Rao et al. (1992) and Jha and Ohri (1996). In the present study, seed protein produced several sequences whose pattern differences enabled various genotypes to be distinguished. Variation was observed in all three regions investigated, with major differences in region III where six bands were recorded and five were polymorphic. Moller and Spoor (1993) suggested five regions for *Lolium* spp. and observed major differences in the B, C and D regions. Previously, Ferguson and Grabe (1986) and Murphy et al. (1990) indicated the potential of electrophoresis techniques for determining the extent of genetic variation in crop germplasm. Electrophoresis adds information to taxonomy, and should not be disassociated from morphological, anatomical and cytological observations (de Vries, 1996).

In black gram, SDS-PAGE cannot be used for identification of various genotypes on the basis of intraspecific variation, because some of the accessions that differed on the basis of characterization and evaluation exhibited similar banding patterns; this technique might be used to study inter- rather than intraspecific variation in *Vigna* spp. (Ghafoor et al., 2002). Genotypes with similar banding patterns are suggested to be studied for detailed agronomic and biochemical analyses, including 2-D electrophoresis and DNA markers, for better management of the genebank (Celis and Bravo, 1984; Beckstrom-Sternberg, 1989). Another option could be the use of larger scale gels or gradient gels, for finer resolution of genetic diversity.

The black gram germplasm evaluated in the present study exhibited significant variation for most of the quantitative characters. Although variation was observed for total seed protein, it was not enough to use for studying inter-accession diversity; these investigations need to be extended to incorporate other biochemical markers in black gram. PCA and cluster analyses demonstrated their validity in establishing genetic diversity. These statistics based on quantitative characters proved more reliable than SDS-PAGE, but no clear geographic relationship was found in any case, although a low-level association between quantitative traits and geographic origin was observed.

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