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INVESTIGATION OF GENETIC DIVERSITY IN BLACK GRAM [*VIGNA MUNGO* (L.) HEPPER]

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Abstract

In the present investigation 34 Pakistani Black gram cultivars were evaluated through morphological traits, SDS-PAGE analysis and random amplified polymorphic markers (RAPD) which revealed considerable amount of genetic diversity in this species. Among the morphological traits, dry pod weight (71.79%), number of branches per plant (51.16%) and biological yield (50.12%) showed highest level of coefficient of variation. Percent frequency distribution also showed highest level of genetic diversity. Positive significant correlation was observed between numbers of pods per plant and dry pod length (p=0.01); grain yield and number of seeds per plant (p=0.02), and 100 seeds weight and biological yield (p=0.04). A total of 20 cultivars were identified and characterized on the basis of maximum and minimum value for 10 quantitative traits. The combinations of 20 cultivars are having practical application for breeding program as well as for direct selection of super cultivar. Among the biochemical and molecular makers, RAPD marker showed high level genetic similarity difference (0.00–4.00) as compared to SDS-PAGE (0.00–2.45). RAPD analysis is considered the best option for determining Black gram genetic diversity pattern and Gene Bank management.

Introduction

Black gram (V*igna mungo* (L.) Hepper) is a self pollinating and widely cultivated grain legume (Naga *et al*., 2006). It is one of the important pulse crop, grown throughout Pakistan. The crop is resistant to adverse climatic conditions and improves the soil fertility by fixing atmospheric nitrogen in the soil. Black gram is one of the rich sources of vegetable protein and some essential mineral and vitamins for human body.

Several qualitative and quantitative biochemical and molecular marker loci encoding storage proteins, isozymes or restriction fragment length polymorphism are currently available for measuring variation between closely related germplasm sources (Sultana & Ghafoor 2008). Morphological characters facilitate in the identification and selection for the desirable traits (Nisar *et al*., 2008). This availability of genetic variability for a given character is a prerequisite for its upgrading by systematic breeding programme. Valuable information on diversity can also be furnished by studying the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of seed storage proteins and the random amplified polymorphic DNA (RAPD) of the desired genotypes. These are considered as practical and reliable methods because seed storage proteins and nucleotide sequences are largely independent of environmental fluctuations (Iqbal *et al*., 2005). No single method is adequate for assessing genetic variation because the different methods sample variation at different level and difference in their power of genetic resolution, as well as in the

quality of information content (Sultana & Ghafoor, 2008). As the germplasm is a vital source in generating new plant types having desirable traits. It helps in increasing crop quality and production which improves the level of human nutrition. The present study was therefore carried out to study the genetic diversity on the basis of morphological traits in conjunction with the SDS-PAGE and RAPD marker analysis which may provide useful information for the use of genotypes in different breeding experiments.

Materials and Methods

The present study was conducted for the estimation of genetic diversity among 34 elite genotypes of *Vigna mungo* on the basis of morphological characteristics, molecular and biochemical markers.

Morphological studies: Thirty-four elite genotypes were obtained from the Pulses Program, National Agriculture Research Institute, Islamabad. Plants were grown in three lines of 5.0m with inter and intra row spacing of 75.0cm and 15.0cm, respectively. Proper irrigation and cultural practices were followed throughout the crop season to get healthy and vigorous plants. Plants were harvested at physiological maturity and data were recorded on 10 randomly selected plants from the middle row. Morphological traits studied included were; plant height^{-1} (cm), Biomass^{-1} (g), Number of Branches^{-1}, Number of Pods^{-1}, Seeds/pod, Pod Length^{-1} (cm), Pod Weight^{-1} (g), Grain Yield^{-1} (g), 100-Seed Weight^{-1} (g) and Harvest Index%.

SDS-PAGE studies: To determine the variation in seed storage proteins electrophoresis was carried out for 34 genotypes, in the discontinuous Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) system of Leammli (1970) using 12.25% (w/v) stacking gel. The electrophoresis and staining/de-staining procedure was carried out following Nisar *et al*., (2007). The data was recorded on the basis of presence and absences of protein bands i.e., 1 for the presence and 0 for the absence of bands. The intensity of band was considered as major and minor bands i.e., the high intensity glowing bands as major and low intensity glowing bands as minor. Cluster analysis was carried out using software STATISTICA (www.statsoft.com).

RAPD markers: Total genomic DNA was extracted from dried seeds of each cultivar according to the method described by Kang *et al*., (1998) with minor modifications. A 400µl of extraction buffer (*200mM Tris-HCl* (pH 8.0), *25mM EDTA*, *200mM NaCl*, *0.5% SDS*), Proteinase K (*50µg*) was added to each tube containing seeds and incubated at 37ºC for 1 hour. Seeds were ground in the buffer with a glass rod. Then 400µl of 2% CTAB solution (*100mM Tris-HCl* (pH 8.0), *20mM EDTA* (pH 8.0), *1.4M NaCl*, 2% *CTAB* (w/v), 1% *PVP* "polyvinylpyrrolidone 40,000) was added. It was extracted by using chloroform: isoamyl alcohol (24:1) with 5% phenol. Centrifuged at 12,000rpm for 10 min at 4ºC and supernatant transfer into new tubes and⅔ volume of Isopropanol was added. Tubes Centrifuged at 12,000 rpm for 5 minutes and supernatant removed. Air dried DNA pellet was re-suspended for 5-10 minutes. Remove RNA by adding 1µl of RNase (*10mg/ml*).

The DNA was quantified (20ng/µl) with the help of spectrophotometer at a wavelength of 260 and 280nm using NanoDrop ND-1000 Spectrophotometer. After standardization of PCR, 20µl reaction mixture containing 1x PCR buffer [10mM Tris HCl (pH 8.3), 50mM KCl], 1.5mM $MgCl_2$, 200µM each deoxynucleotide triphosphate (dNTP), 0.4µM of 10-mer primer (Operon Technologies Inc., Alameda, CA), one unit Taq DNA polymerase and

approximately 20ng of template DNA was found optimum for the amplification of black gram genomic DNA. The thermal cycler was programmed to one cycle of 5 minutes at 94ºC for initial strand separation. This was followed by 45 cycles of one minute at 94ºC for denaturation, one minute at 36ºC for annealing and two minutes at 72ºC for primer extension. Finally, one cycle of 7 minutes at 72ºC was used for final extension, followed by soaking at 4°C. After an initial screen of 20 RAPD primers, seven primers were chosen to detect the polymorphism and produce the banding patterns in black gram cultivars. The experiment was repeated twice to check the reproducibility. Binary data was recorded on the presence and absence of bands for each primer.

Data analysis: Data on morphological traits was analyzed for the determination of simple statistics and correlation among the various character pairs by using the computer software STATISTICA 6.0. Using numericd data of morphological traits and binary data of both SDS-PAGE and RAPD bands, similarity index was calculated for all possible pair of protein types. To avoid taxonomic weighing, the intensity of bands was not taken into consideration and only the presence of bands was taken as indicative. Based on results of electrophoresis band spectra, un-weighted pair-group method with arithmetic averages (UPGMA) was calculated of Sneath & Sokal (1973) by using a statistical package, STATISTICA Ver.6.0.

Results

1. Morphological based genetic diversity: For morphological traits coefficient of variation was calculated to check the level of variation among the local cultivars of black gram. Among the 10 quantitative traits plant height contributed 25%, number of branches per plant 51.16%, dry pod length 9.02%, number of seeds per pod 8.28%, dry pod weight 71.79%, biological yield 50.12%, grain yield 12.3%, and number of pods per plant 8.28%, 100 seed weight 3.74% and harvest index 31.63% of coefficient of percent variation (Table 1).

A total of 20 cultivars were identified and characterized on the basis of maximum and minimum value for 10 quantitative traits (Table 2). Ten Cultivars ICM-710, NCH9-5, VH9440034-2, VH9440034-3, VH9440034-9, VH9440034-1, NCH3-4, NCH9-2, 9092 and VH9440034-9 were grouped in the combination of minimum level; and similarly, 10 VH9440039-3, VH9440034-7, NCH9-3, VH9440034-9, VH9440034-1, 9092, VH9440023-1, VH9440034-9, VH944003-7, and VH9440023-2 grouped in combination of maximum level of plant height, number of branches/plant, dry pod length, number of seeds/pod, dry pod weight, number of pods/plant, 100 seeds weight, biological yield, grain yield and harvest index respectively (Table 2).

On the basis of class interval, five groups were made in order to show frequency distribution with of 10 quantitative traits. Frequency distributions for the traits were calculated to classify the cultivars into different categories (Fig. 1). It indicated that a maximum of 65% of the populations were having ≥ 38.08 to ≤ 50.08cm plant height, while 73.5% ranged in ≥ 4 to ≤ 7.4 number of branches/plant. Furthermore, 41.2% of the total germplasm ranged in ≥ 3.26 to ≤ 3.63cm dry pod length and 29.4 % were ranged ≥ 4.73 to ≤ 5.04 number of seeds/pod. Out of 34 cultivars, 30 were ranged ≥ 0.24 to ≤ 0.58g of dry pod weight, while similarly 22 ranged ≥ 4.36 to ≤ 9.07g of total biological yield. A maximum of 29.4% cultivars ranged ≥ 8.84 to ≤ 9.73g of grain yield and 41.2% ranged ≥ 20.90 to ≤ 27.63g harvest index (Fig. 1).

Table 1. Basic statistics for 10 quantitative traits of 34 cultivars of black gram.

	Traits	Mean	Max.	Min.	Range	Variance	SE	SD	CV%
1.	Plant height	46.7	86.12	26.06	60.06	137.83	2.01	11.74	25%
2.	No of branches	6.92	21	4	17	12.53	0.61	3.54	51.16%
3.	Dry pod length	3.77	5.12	3.26	1.86	0.12	0.06	0.34	9.02%
4.	No of seeds/ pod	5.07	6	4.4	1.6	0.18	0.07	0.42	8.28%
5.	Dry pod weight	0.39	1.95	0.24	1.71	0.08	0.05	0.28	71.79%
6.	Biological yield	9.26	27.94	4.36	23.58	21.53	0.8	4.64	50.12%
7.	Grain yield	10.02	12.45	7.92	4.53	1.52	0.21	1.23	12.3%
8.	No. of pods/plant	14.32	76	5.4	70.6	135.02	2	11.62	8.28%
9.	100 seed weight	4.55	5	4.09	0.91	0.03	0.03	0.17	3.74%
10.	Harvest index	24	41.12	7.4	33.72	57.61	1.3	7.59	31.63%

SE= Stander error, SD= Stander deviation, CV%= Coefficient of variance %

Table 2. Minimum and Maximum value of 10 quantitative characters in 34 varieties of black gram.

S. No.	Traits	Minimum	Maximum
1.	Stem length (plant height)	ICM-710 (26.06cm)	VH9440039-3 (86.12cm)
2.	No of branches	NCH9-5 (4)	VH9440034-7 (7.4)
3.	Dry pod length	VH9440034-2 (3.26cm)	NCH9-3 (5.12cm)
4.	No of seeds/ pod	VH9440034-3 (4.4)	VH9440034-9 (6)
5.	Dry pod weight	VH9440034-9 (0.24g)	VH9440034-1 (1.95g)
6.	No. of pods/ plant	VH9440034-1 (5.4)	9092 (76)
7.	100 seed weight	NCH3-4 (4.09g)	VH9440023-1 (4.98g)
8.	Biological yield	NCH9-2 (4.36g)	VH9440034-9 (27.94g)
9.	Grain yield	9092 (7.92g)	VH944003-7 (12.29g)
10.	Harvest index	VH9440034-9 (7.4)	VH9440023-2 (41.11)

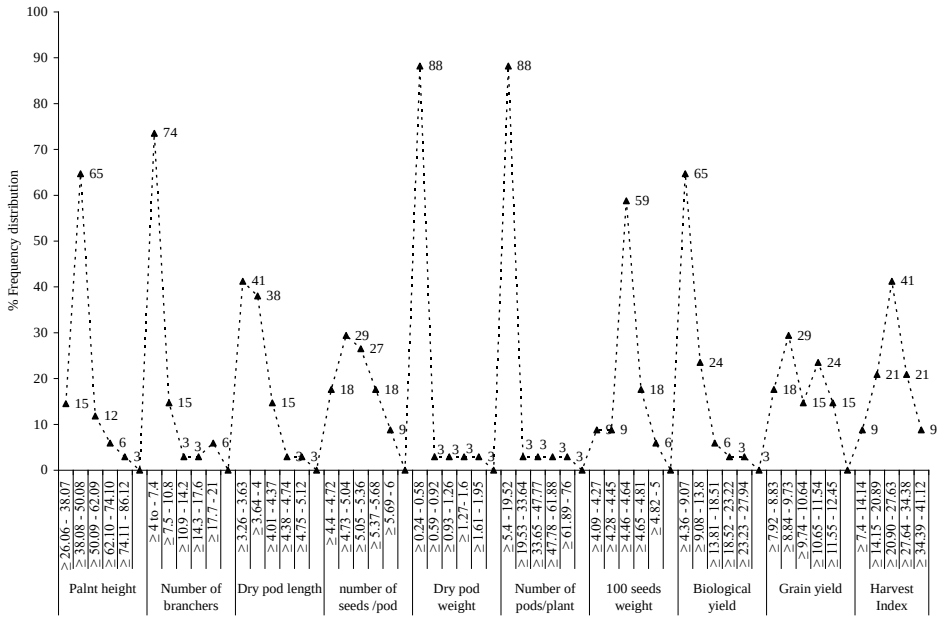

Fig. 1. Percent frequency distribution in each quantitative characters.

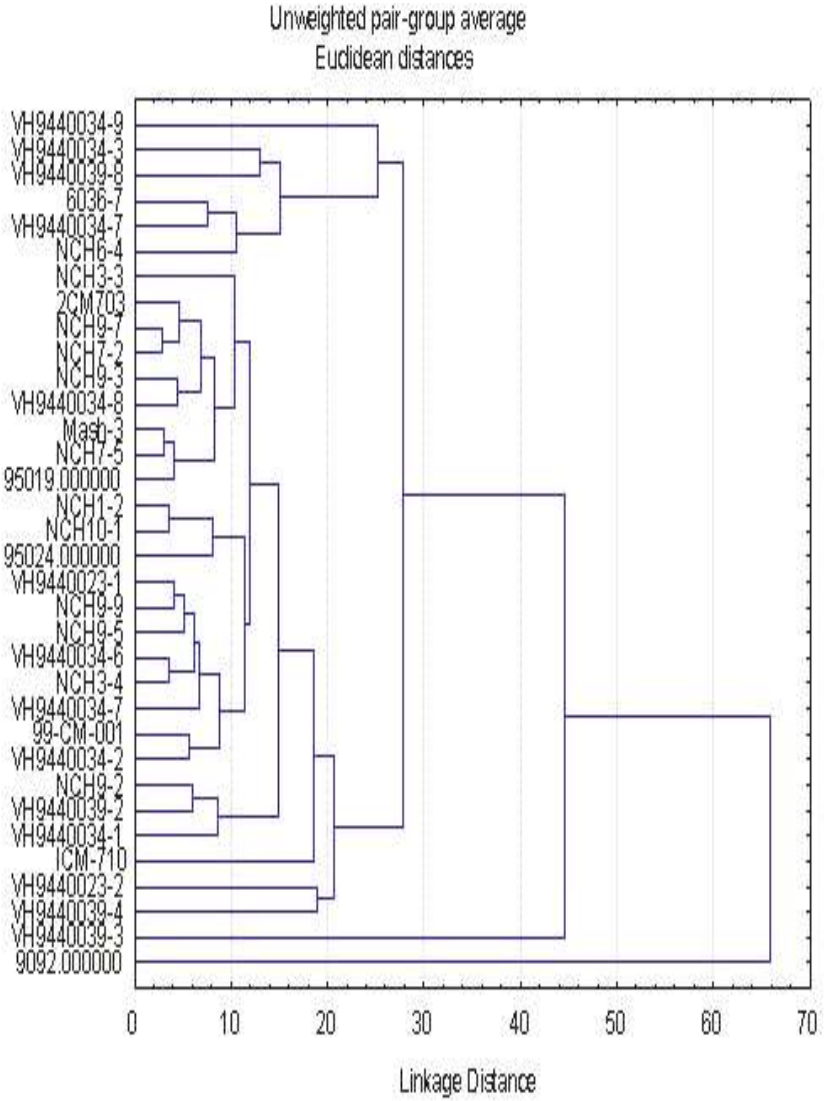

Fig. 2. Cluster analyses showing the relationship among 34 cultivars of black gram using 10 quantitative traits.

Morphological data of commercial varieties of black gram were also analyzed using cluster analysis (Fig. 2). Phenogram based on Euclidean distance coefficients using 10 quantitative traits placed 34 varieties into four main groups. Cluster 1 sort seven cultivars (VH9440034-9, VH9440034-3, VH9440039-3, VH9440039-8, 6036-7, NCH6-4, VH9440034-7), twenty-one varieties (NCH3-3, 2CM703, NCH1-2, NCH10-1, VH9440023-1, VH9440034-6, NCH9-3, ICM-710, Mash-3, NCH9-5, 95024, 99-CM-001, NCH3-4, VH9440034-8, NCH9-7, NCH9-9, VH9440034-2, NCH7-5, VH9440034-7, 95019, NCH7-2) fall in Cluster 2, one 9092 in Cluster 3 while five cultivars (VH9440023-2, NCH9-2, VH9440039-4, VH9440034-1, VH9440039-2) were grouped in Cluster 4.

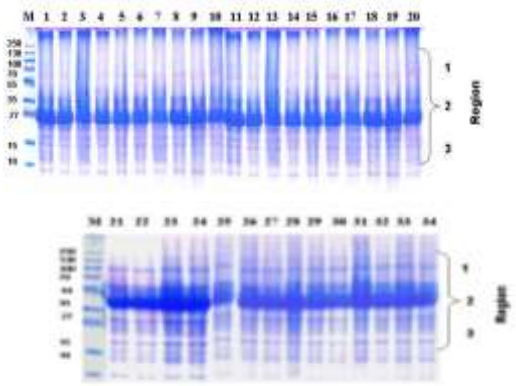

Fig. 3. SDS-PAGE banding patterns of 34 cultivars of black gram. The lane M represents molecular weight marker; (1) VH9440034-9 (2) VH9440034-3 (3) VH9440039-3 (4) VH9440039-8 (5) NCH3-3 (6) 2CM703 (7) 6036-7 (8) NCH6-4 (9) NCH1-2 (10) NCH1-2 (11) VH9440023-3 (12) VH9440034-6 (13) NCH9-3 (14) ICM-710 (15) VH9440023-2 (16) Mash-3 (17) NCH9-5 (18) 95024 (19) NCH9-2 (20) 99-CM-001 (21) NCH3-4 (22) VH9440034-8 (23) VH9440039-4 (24) NCH9-7 (25) VH94440034-1 (26) VH9440034-7 (27) NCH9-9 (28) VH9440034-2 (29) NCH7-5 (30) 9092 (31) VH9440039-2 (32) VH9440034-7 (33) 95019 (34) NCH7-2.

2. Biochemical based genetic diversity: SDS-PAGE of 34 cultivars was carried out; the protein profile of each cultivar was subjected to cluster analysis (Fig. 3). Cluster analysis of 34 cultivars of black gram total seed storage protein was performed on the results basis of SDS-PAGE using the software STATISTICA to find out diversity among the accessions. The results of the cluster analysis were presented in the dandrogram on the basis of linkage distance .The cluster diagram revealed two major groups. Group A consist of cluster of four cultivars (VH3440023-1, VH9440034-6, NCH9-3 and ICM-710). Group B consist of two sub groups B1 and B2. Group BI has two cultivars (VH9440023-2 and NCH9-5). Further the B2 comprised of two subgroups BII and BIIb, whereas BIIb consist of two cultivars Mash 3 and VH9440023. BIIa consist of BIIaI and BIIaII. BIIaI consist of 17 cultivars (VH9440034-9, NCH3-3, 6036-7, VH90039-4, NCH9-9, VH9440034-2, NCH7-5, VH9440034-7, 95019, NCH7-2, NCH6-4, NCH1-2, VH9440034-1, 9092, VH9440039-2, 95024 and 99CM-001). Subgroup BIIaII consist of 9 cultivars (VH9440034-3, VH9440039-3, VH9440039-8, 2CM703, NCH9-2, NCH101, NCH3-4, NCH9-7 and VH9440034-7) as shown in Fig. 4.

A similarity matrix based on the proportion of shared bands was used to establish the relationship between the black gram varieties during present investigation. As expected, genetic distances between the accessions were comparatively low. Pair wise estimates of similarity for 34 varieties ranged from 0.00 to 2.45. VH9440034-9, VH9440034-3, 2CM706, NCH10-1, NCH1-2, NCH10-1, VH9440023-2, NCH9-5, 95024, NCH9-2, NCH9-7 and VH9440034-1 were the closest genotypes with the lowest similarity index of 0.00, while the highest similarity index 2.45 were found in VH9440023-1, VH9440034-6 and NCH9-3.

3. Molecular based genetic diversity: In the present study 5 different RAPD primers were run to check the nature of genetic diversity in the present germplasm (Fig. 5). Cluster analysis divides the cultivars into two main groups. Group A consist of one

genotype NCH7-2. Group B is divided into BI and BII. BI consists of genotype 95019. Cluster BII sub divided into BIIa and BIIb. BIIa consist of two accessions, VH9440034-7 and ICM-710.BIIb further divided into BIIb1 and BIIb2. BIIb1 consist of one genotype VH9440034-1, while BIIb2 consist of 29 accessions. As expected from the similarity estimates, cluster analysis placed most of the cultivars close to each other showing a high level of genetic similarity and low level of genetic diversity (Fig. 6).

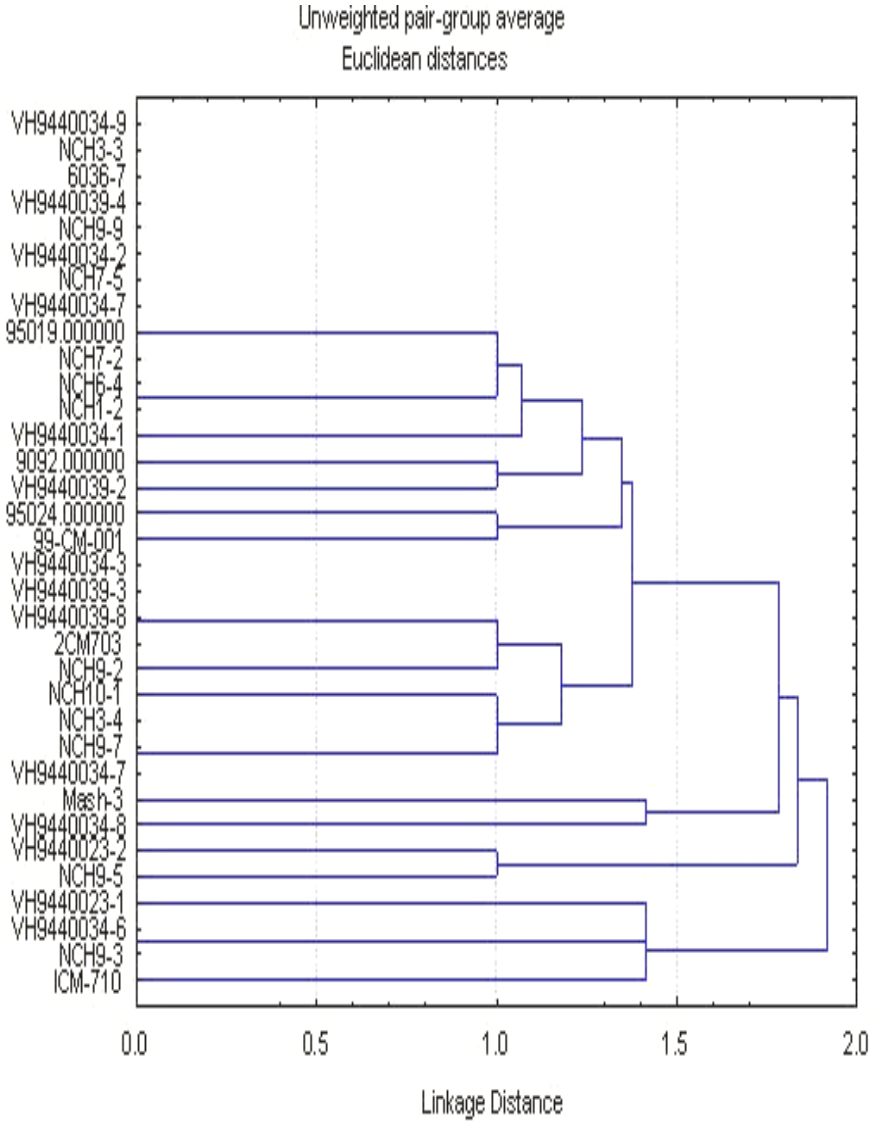

Fig. 4. Cluster analysis showing the relationship among 34 cultivars of black gram using SDS-PAGE banding patterns.

A similarity matrix based on the proportion of shared RAPD fragments was used to establish the level of relationship between the black gram accessions during present investigation. Pair-wise estimates of similarity for 34 accessions were from 0.00 to 4.00. Five closely related genotypes were VH9440023-2, NCH9-5, VH9440034-8, VH9440034-7 and 9092 with similarity index of 2.65. Genotypes of similarity index of 3.00 were NCH3-3, 6036-7, ICM-710, NCH3-4 and VH9440034-2, whereas similarity index of 3.16 observed in three closely related genotypes, VH9440039-3, VH9440039-8 and 95024. Out of 34 cultivars, NCH9-2 and VH9440023-1 were having greatest dissimilarity with all the other tested cultivars.

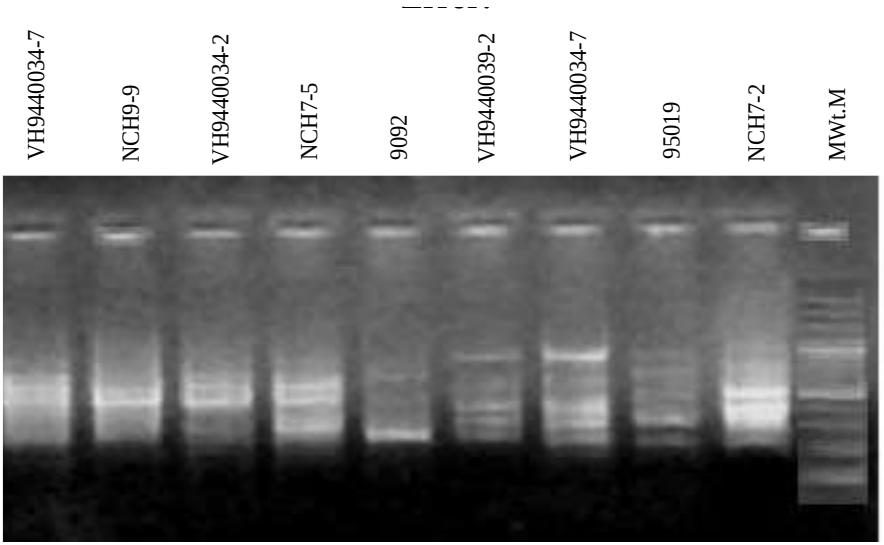

Fig. 5. RAPD banding pattern of 34 cultivars of black gram generated by random primer Z-10.The lane M represents 1-Kb Mass Ruler marker.

Discussion

Variation between and within population of crop species is useful for analyzing and monitoring germplasm during the maintenance phase and predicting potential genetic gain in breeding programs (Sultana & Ghafoor, 2008). Morphological and agronomical characters of the collected germplasm facilitate in identification and selection for desirable traits (Ghafoor *et al*., 2002 & Zahir *et al*., 2008). During the morphological traits > 50% genetic diversity and marked level of frequency distribution was observed in number of branches per plant, dry pod weight and biological yield. Considerable level of > 25% genetic variation was observed in Plant height and harvest index. Since variation is the basis of improvement, during the present study high level of genetic variation was observed for yield contributing traits and can be exploited for yield in breeding program. The traits with low level of genetic diversity required further exploration and collection to enhance the level of diversity. A total of 20 cultivars were identified and characterized on the basis of maximum and minimum value for 10 quantitative traits. The combinations are highly potential for breeding program as well as for direct selection of super cultivar.

Seed protein analysis by SDS-PAGE has proved to be an effective way of revealing the differences (genetic diversity) and relationships between taxa (Nisar *et al*., 2007). The black gram germplasm evaluated in the present study exhibited significant variation for

most of the quantitative characters. Variation in total seed protein are fairly low to (0.00–2.45), use for studying inter-accession diversity. Therefore, these investigations need to be extended to incorporate other bio-chemical markers in black gram. Similarly SDS-PAGE showed low diversity in the storage protein banding patterns of the present material, it should to be broadened through collections and acquisition of germplasm from centre of origin.

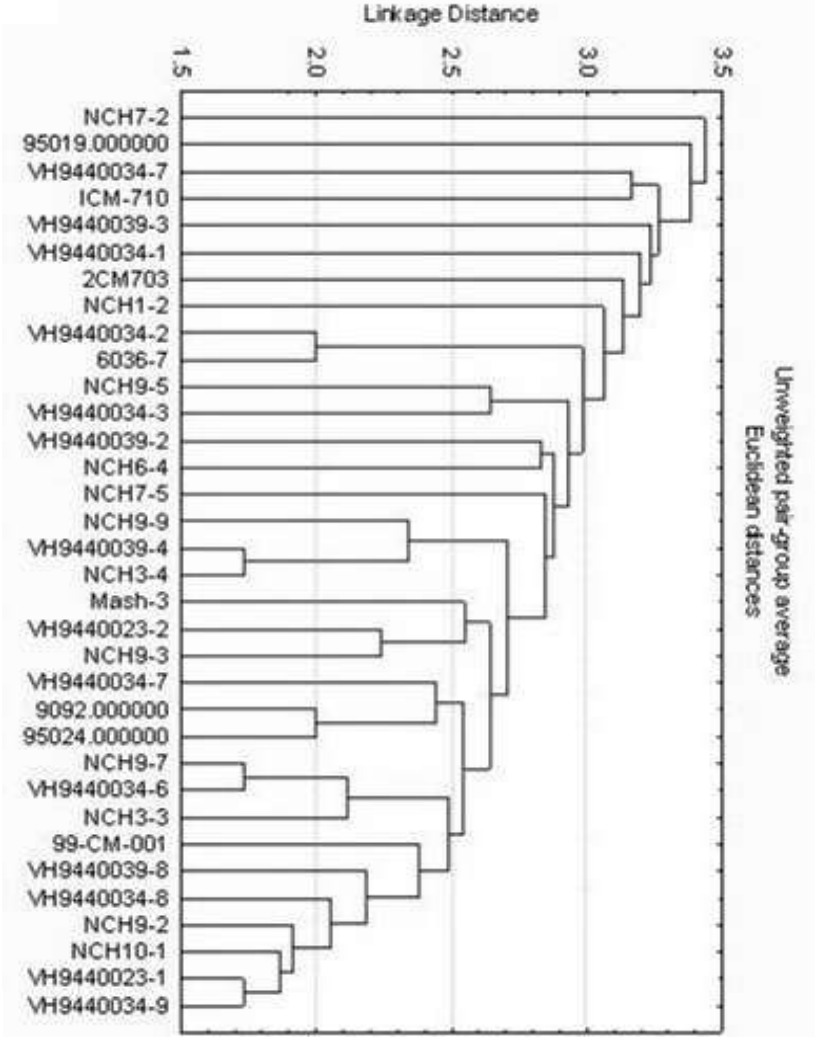

Fig. 6. Cluster analysis showing the relationship and diversity among 34 cultivars of black gram by using RAPD analysis.

RAPD markers found to be important to resolve various levels of inter-and intra-specific polymorphism, which facilitates the assessment of genetic relationships, definition of regional grouping and identification of individual accessions (Virk *et al*., 1995). In RAPD marker availability of unique locus-specific allelic profiles (band/bands), that are accurate and reproducible, are highly potential for fingerprinting and various identification of superior cultivated field Black gram genotypes. In the present study the

number of amplified fragments with random primers ranged from 1 to 5 and varied in size from 300bp to 2000bp. In addition RAPD marker showed high level of different genetic similarity (0.00–4.00) as compared to SDS-PAGE (0.00–2.45). Difference in high level of genetic similarity suggested that RAPD markers may be the best option for genetic diversity investigations. It is widely recognized that the plant with divers pattern for RAPD are selected for further study and to select parents for inheritance or linkage group (Eujayl *et al*., 1998).

On the basis of present investigations it is concluded that morphological traits, SDS-PAGE analysis and random amplified polymorphic markers revealed considerable amount of genetic diversity in Black gram. A total of 20 cultivars were identified and characterized on the basis of maximum and minimum value for 10 quantitative traits. The combinations of 20 cultivars are highly potential for breeding program as well as for direct selection of super cultivar. It is suggested that among the biochemical and molecular makers, RAPD is the best option to expose inter-and intra-cultivars variation and is needed to extend to more germplasm and primers for further study, along with morphological traits analysis.

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