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GENETIC DIVERSITY IN BLACKGRAM [*VIGNA MUNGO* (L.) HEPPER] FOR RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

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Abstract

Thirty seven pure-lines of blackgram were studied for Randomly Amplified Polymorphic DNA (RAPD) markers to investigate diversity. Among 53 primers 36 produced polymorphic fragments in blackgram, hence could be used for investigating genetic diversity. Ninety four markers were observed from 11 primers and out of these 72% were polymorphic. The genotypes acquired from Korea, Afghanistan and AVRDC grouped together, whereas genotypes from Pakistan and India were scattered. The RAPD markers were found useful for studying genetic diversity but clustering did not exhibited indication for agronomic performance, whereas quantitative traits contributed more towards agronomic performance. Cluster revealed that only a portion of genetic diversity has been exploited for blackgram improvement that should broaden involving diverse parents from various clusters.

Introduction

Among molecular techniques available for assessing genetic variability, relatedness and structure among crop germplasm collections, randomly amplified polymorphic DNA (RAPD) provide powerful and reliable tool for discerning variations within crop germplasm and for studying evolutionary relationships (Gepts, 1993; Williams *et al.,* 1993; Jatoi *et al*., 2008; Vir *et al.,* 2010). Molecular biology introduced a method for genotypic identification that is superior as compared with agronomic and seed proteins and can also be used for mapping purposes (Paterson *et al*., 1991; Kiss *et al*., 1993; Eujayl *et al*., 1997; Souframanien *et al.,* 2008). The RAPD requires no previous sequence information for the fingerprinting of cultivar genomes rather these can be converted to specific markers (Gresshoff, 1998). These have been used widely for estimating genetic variation at the population level and among closely related species (Rafalski *et al.,* 1991; Eujayl *et al*., 1998; Wang *et al*., 2008). The technique has been found successful to resolve various levels of inter and intra-specific polymorphism, which facilitates assessment of genetic relationships, definition of regional grouping and identification of individual accessions (Strelchenko *et al.,* 1999; Dikshit *et al*., 2007; Yi *et al*., 2008). Since the genetic differences are reflected as the presence or absence of RAPD fragment and a huge number of bands can be obtained in a limited time, whereas seed protein markers do not give polymorphism for genetic diversity within *Vigna mungo* (Ghafoor *et al.,* 2002).

Blackgram or mash [*Vigna mungo* (L.) Hepper] is an important summer pulse crop of many South Asian countries including Pakistan, India, Nepal, Bangladesh, Thailand, Philippines and Korea. It is cultivated under a wide range of agro-ecological zones mainly of rainfed nature. Among pulses, it is the least researched crop and no international centre of CGIAR system has this crop on its mandate (Anon., 1976). Although it has been identified as a potential crop in a number countries, but no systematic molecular research information is available except few reports worldwide, whereas in Pakistan no such study has been reported. The objectives of this study were to determine genetic diversity in blackgram on the basis of RAPD markers.

Materials and Methods

Thirty seven pure-lines of blackgram including one check (Mash 1) were investigated for genetic diversity on the basis of RAPD and this sample was derived from the material that we have already published (Ghafoor *et al*., 2001). The seeds of each genotype were disinfected with 99.95% ethanol, washed with distilled water to avoid contamination and then planted between filter papers in the Petri plates and were placed in the dark at room temperature (RT). After the emergence of plumule, the plates were shifted to the incubation room at 30°C under normal light. After 5 days of transfer, the first 2 leaves were cut with disinfected scissors and dipped in liquid nitrogen (LN) for 10 minutes. After this, leaf samples were preserved in -80°C till the extraction of DNA.

Thirty mg of primary leaves collected and frozen at -80°C were ground in LN, mixed vigorously with extraction buffer (20% SDS, mercaptoethanol) and incubated at 65°C for 10 minutes. Proteins of the solution were removed by the addition of phenol/chloroform/isoamylealcohol (25:24:1). The DNA was precipitated with isopropanol by incubation at -20°C for 30 minutes, and then centrifuged at 12,000 rpm for 15 minutes. The pellet was washed with 70% ethanol, dried and then suspended in 1 ml of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0). The DNA was digested with 1 µg RNase and incubated at 37°C to remove the RNA from the solution. The DNA quantity was estimated with spectrophotometer for dilution for PCR. The DNA thus obtained was diluted to 100, 50, 10, 5 and 2.5 ng/µl to confirm the actual dilution for PCR. Using the mini-gel method the appropriate DNA amount (a glowing white thread thickness) for PCR amplification was determined by visual comparison with lambda DNA of known concentration (Sambrook *et al*., 1989). The PCR was performed in the 0.5 ml reaction tubes with 5 µl of DNA

solution containing 2.5 ng of genomic DNA, 1 µl of primer, 0.2 µl of 20 mM dNTP, 2 µl of 10X buffer, 1.6 µl $MgCl_2$ and 0.2 µl of *Taq Polymerase*. The volume in the tube was made 20 µl by adding 10 µl of H_2O. Twelve mer Operon oligonucleotide primers were used for PCR. The reaction mixture was overlaid with a drop of mineral oil to avoid evaporation. Amplification reaction was carried out at the Perkin Elmer thermocycler with 94^o C for 1 minute, 30^{o}C for 1 minute and 72^{o}C for 2 minutes for 45 cycles. Prior to storage at 4^o C annealing was completed at 72^{o}C. The detailed protocol for DNA extraction in blackgram and PCR amplification has been presented by Ghafoor, (1999).

The amplified fragments were recorded after the electrophoresis on 13% Polyacrylamide Gel , with the composition of 33.06 ml H_2O, 9.6 ml Acrylamide (39:1), 4.8 ml of 10 X TBE, 0.48 ml of 10% fresh APS and 0.06 ml of Tetramethylethylenediamine (TEMED). The solution was mixed thoroughly and poured into the glass plates to let the gels polymerise. Gels were allowed for pre loading run at 150 constant voltages for 30 minutes. Eight µl of each sample was applied in the well and electrophoresis was conducted at 150 constant voltages for 70 minutes. Five µl of the molecular marker X 174/*Hae* III digest was used. After the completion of electrophoresis, the gels were stained in Ethidium bromide (200 ml TE and 10 µl Ethidium bromide). Amplification was observed on the UV illuminator and Polaroid 667 was used for photographs.

The RAPD behaves as dominant markers thus they tend to be bistate (present-absent) type scoring (Clark & Lanigan, 1993). Photographs of Ethidium bromide stained polyacrylamide gels were used to score RAPD markers. Each DNA fragment amplified by a given primer was treated as a unit character and the RAPD fragments were scored as present (1) or absent (0) for each of the primer-genotype combinations. Since DNA samples consisted of a bulk sample of DNA extracted from individual plants, a low intensity for any particular fragment may be explained by the lesser representation of that specific sequence in the bulk sample of DNA. Therefore, the intensity of the bands was not taken into account and the fragments with the identical mobility were considered to be the identical fragments. Only major bands were scored and faint bands were not considered. The molecular size of the amplification products was calculated from a standard curve based on the known size of DNA fragments of a marker X 174/*Hae* III digest. Pair-wise comparisons of the genotypes based on the presence or absence of unique and shared amplification products were used to generate similarity coefficients. DNA band shared by all the genotypes were excluded from the data analysis since they were not informative.

The resulting similarity coefficients were used to evaluate relationships among the genotypes with a cluster analysis using an un-weighed pair-group method with arithmetic averages (UPGMA) and then plotted in the form of a dendrogram using computer software STATISTICA Version 7.0 for windows Vista. The first two factors as observed by analyzing data through principal component analysis were plotted for genotypes under study using computer software "SPSS" for windows.

Results

Fifty three 12 mer primers from *Operon Technology* were studied to find the initial results for amplification. These primers were tested using 4 genotypes (41117, PL-2, MM 5-60, 45737) for DNA amplification initially and among these, 46 were amplified and out of these, 36 exhibited polymorphism and hence could be used for DNA fingerprinting (Table 1). Finally, out of 36 primers, 11 were used for RAPD analysis of all the 37 genotypes of blackgram. Each of the 11 primers varied greatly for their ability to resolve variability among 37 genotypes. Some of the primers generated several markers and were able to show a high level of genetic diversity, while others produced few markers and detected little variability (Fig. 1). The Agrose gel amplified by the primer OPD 20 presented as figure indicated a considerable amount of variation at DNA level that could be utilized for investigation of genetic diversity and genotyping of blackgram germplasm. Eleven primers generated a total of 94 reproducible and score able amplification products across all the genotypes, out of which 69 (72 %) fragments were polymorphic in one or other genotypes. The rest of the 25 bands were monomorphic for the present material (Table 2). Among the primers, OPD 20 generated eleven polymorphic fragments that were followed by the OPF 22 with ten polymorphic fragments. The primer OPA 73 produced two markers and one was polymorphic. The primers, OPF 22 and OPC 96 generated all the polymorphic fragments. The agronomic data on the germplasm used for DNA markers in the present has been published by Ghafoor and Arshad (2008a), and we used the DNA marker data in comparison with agronomic data and no specific RAPD marker was observed related to agronomic traits or geographic indicator. Cluster analysis based on UPGMA suggested the existence of seven clusters (Fig. 2). The genetic diversity was not conspicuous but variance was observed at micro level and all the genotypes were different from each other although the number of fragments varied accordingly and the clusters were observed at 85% similarity index. The Clusters I (45851), II (41096), IV (45737), V (41016) and VI (PL-2) consisted one genotype in each case, cluster III consisted 2 genotypes (45855, 45846), whereas all the other genotypes were in cluster VII. Out of five genotypes comprising one cluster, three were exotic that differed significantly from the rest of the genotypes. Fig. 3 presents scatter diagram based on first two factors. The genotypes from Korea, Afghanistan and AVRDC were observed grouping together, whereas genotypes from Pakistan and India were scattered. Both the genotypes (MM 5-60 and MM 33-40) from AVRDC were grouped together in cluster diagram as well as scattered diagram indicating the linear relationship between these numerical taxonomic techniques. It was evident from RAPD analysis that it could resolve genetic diversity better even at minimum level.

Table 1. Primers used for preliminary DNA amplification in black gram.

Sr. No.	Primer	Sequence (5′ to 3′)		Sr. No.	Primer	Sequence (5′ to 3′)	
1.	D 20	AAGACCCTACGA	+/-	28.	C 54	ATGCAGAAGTCT	+
2.	D 10	AGGGCCATGATA	+/-	29.	C 95	GTCCACGTGAAG	+
3.	D 40	TCCGAGTATCTG	+/-	30.	C 17	GTTATGCAAGGG	+
4.	F 30	GGAGAGGAAATG	+/-	31.	C 61	AAGAGGGTTGAC	+
5.	F 21	AGCAACAATCCG	+/-	32.	C 75	GATGGTGACGAA	+/-
6.	F 16	ACTAACCTGGAC	+/-	33.	C 85	ACTTTGACAGCG	+/-
7.	D 69	CGCTCCAAATCA	+/-	34.	C 27	GAGGACGTTAAA	+/-
8.	D 91	CTGAAGTATCCC	-	35.	C 67	CCAAGATCCATT	+/-
9.	D 76	CACTTCAACCAG	+/-	36.	C 71	TTCAACATCGAC	+/-
10.	D 65	TACAGCCACTTG	+/-	37.	D 25	GCCATCCGTACA	+/-
11.	D 20	AAGACCCTACDA	+/-	38.	D 45	AGGGTCGTTGCA	+/-
12.	C 20	ACTCACAAATTG	+/-	39.	C 32	TTGCCTCGAGCT	-
13.	C 19	CAGGATGACCTA	+	40.	C 39	CCTAAGGGGTCT	+/-
14.	C 24	AACGAGCAGAAC	+/-	41.	C 78	CTCGAAACGCGT	-
15.	C 26	CACGTTATCGCA	+/-	42.	C 91	CACCTGGCATGG	+/-
16.	C 41	CAGACAGGGTAT	+/-	43.	Z 10	CCCGTGCACGGA	-
17.	C 42	AAGGTCGAACGT	+	44.	C 63	ACTGTTATAACG	-
18.	C 44	GGCAACATAGTA	+/-	45.	F 04	TTGAGTAGTTGC	-
19.	C 92	ATCGACGGAGAA	+/-	46.	D 86	ATGGGCCAGAAG	+/-
20.	C 93	GTCACTCGGATA	+	47.	D 87	GACGCCTATGTC	+/-
21.	C 96	ACCAAGAAAGGG	+/-	48.	D 89	CAGTCTAAGCGG	+/-
22.	C 97	AAGACGGTGGTA	+/-	49.	F 48	GTCCTGCTAACC	-
23.	C 98	ACCAACGTGTAC	+/-	50.	A 73	AGCACTAAATCT	+/-
24.	F 22	AAGATCAAAGAC	+/-	51.	B 32	ATCATCGTACGT	+/-
25.	C 48	ATGAGTGGACAC	+	52.	B 28	GGACAAGTAATG	+/-
26.	C 72	AGATTGCAAGAA	+	53.	D 67	AACACCTCGATC	+/-
27.	C 50	CTTCCTTGGTCA	+				

The sign "+" represents amplification of primer, "-" no amplification and "+/-" represents polymorphism obtained by the primer.

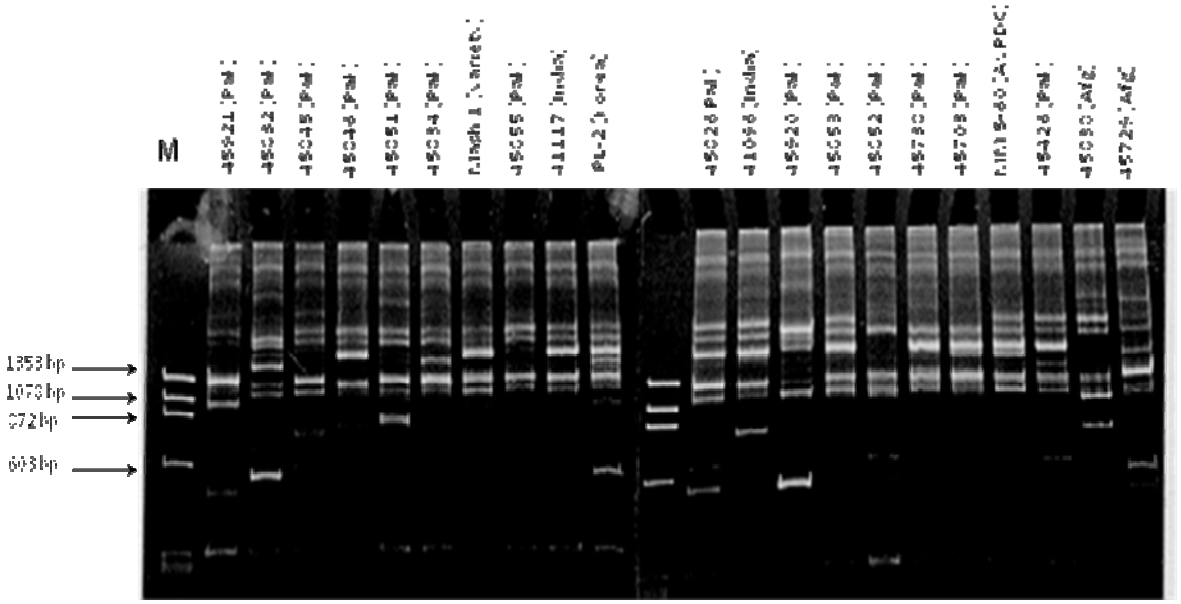

Fig. 1. The RAPD fragments with "OPD 20" [5 –AAGACCCTACGA – 3] among *Vigna mungo* genotypes. The molecular marker X 174/Hae III digest was used. The country of origin, i.e., "Pak" represents Pakistan, "Afg" represents Afghanistan and AVRDC is Asian Vegetable Research and Development Centre, Taiwan.

Table 2. The RAPD fragments observed by using eleven primes in 37 genotypes of blackgram.

Primer	Sequence (5′ to 3′)	Polymorphic bands for molecular weight (bp)					Polymorphic bands	Monomorphic bands	Percent polymorphism
		>1353	>1078	>872	>603	<603			
OPF 22	AAGATCAAAGAC	6	1	0	2	1	10	0	100
OPD 69	CGCTCCAAATCA	2	0	0	3	3	8	4	67
OPF 21	AGCAACAATCCG	2	0	0	3	3	8	4	67
OPA 73	AGCACTAAATCT	0	0	0	0	1	1	1	50
OPC 92	ATCGACGGAGAA	2	1	0	1	1	5	4	56
OPD 20	AAGACCCTACGA	5	3	3	0	0	11	1	92
OPD 67	AACACCTCGATC	3	1	0	2	0	6	5	55
OPC 26	CACGTTATCGCA	4	0	0	0	0	4	1	80
OPC 96	ACCAAGAAAGGG	3	0	2	2	0	7	0	100
OPC 67	CCAAGATCCATT	1	0	1	1	0	3	2	60
OPB 32	ATCATCGTACGT	3	1	1	1	0	6	4	60
Total		31	7	7	15	9	69	25	72

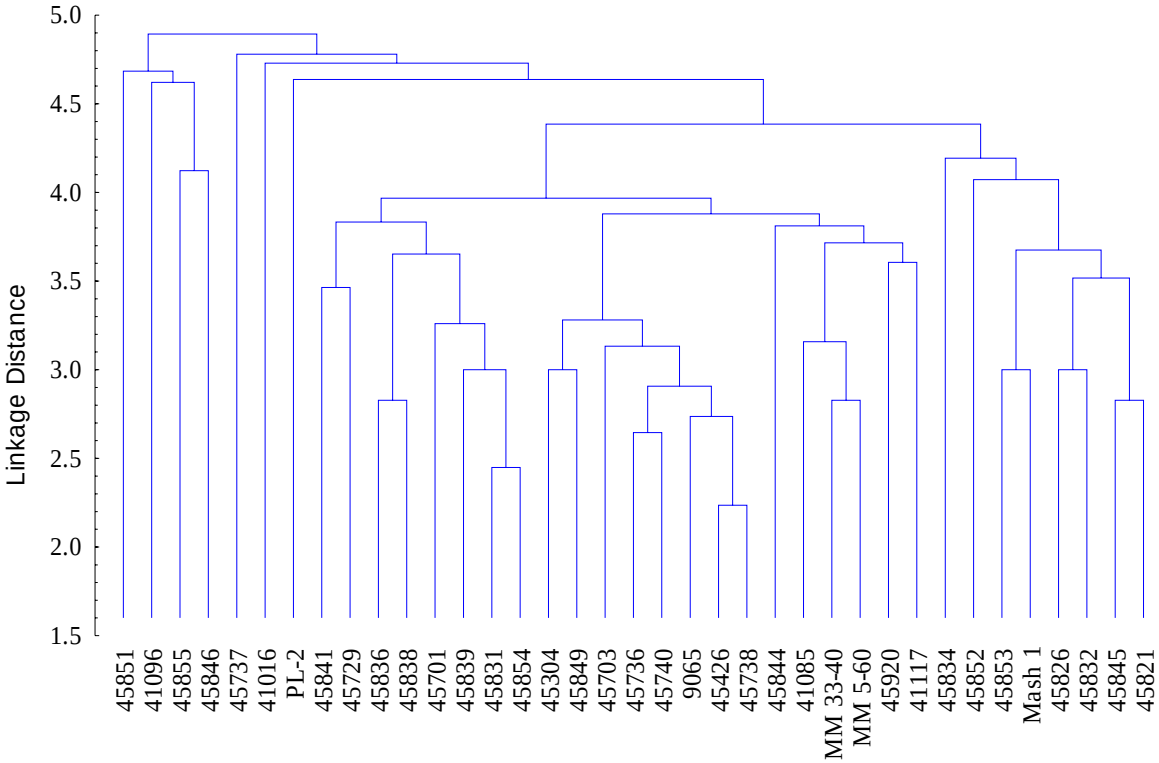

Fig. 2. Dendrogram on random amplified polymorphic DNA Markers (69 fragments) in *Vigna mungo*.

Discussion

RAPDs have been shown to provide a level of resolution equivalent to RFLPs for determining genetic relationships among genotypes (dos Santos *et al.,* 1994). This technique has been found important in *Vigna mungo* to resolve intra-specific polymorphism, whereas low variation in *Vigna reflexo-pilosa* was reported by Vaughan *et al*., (2000). This facilitates assessment of genetic relationships, definition of regional grouping and identification of individual genotypes (Link *et al*., 1995; Virk *et al*., 1996; Chaitieng *et al*., 2006). Arulbalachandran (2010) investigated genetic variation among four mutants of blackgram using 20 random primers and observed 0.19 genetic distances among five genotypes. Many primers used in this study generated polymorphic fragments indicating the usefulness of RAPD analysis to disclose DNA polymorphism in this taxon (Kaga *et al.,* 1996; Xu *et al*., 2000). Primers generated genotype-specific amplification products and thus preliminary mapping in blackgram is suggested. The contributions of genetic markers to gene mapping or to plant breeding have been reviewed by Tanksley (1983), Stuber (1992), Dudley (1993) and Souframanienv (2008). Shafique *et al*., (2011) reported genetic diversity in blackgram based on seed protein profiling and RAPD with presenting percent diversity for both the parameters. The RAPD marker were reported better for investigation of intra-specific genetic diversity in blackgram as SDS-PAGE and considered RAPD better for determining genetic diversity and genebank management.

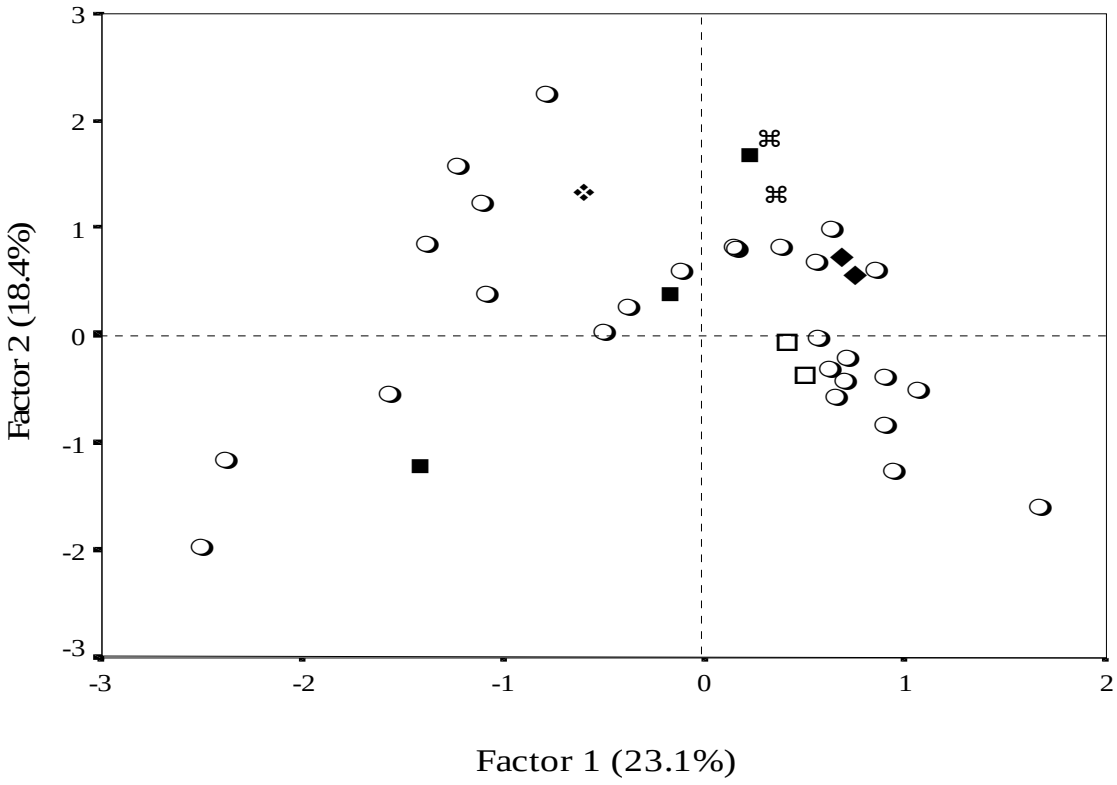

Fig. 3. Scattered diagram of 37 genotypes of black gram based on random amplified polymorphic DNA markers.
❍-Pakistan, ■- India, ❑-AVRDC, ⌘-Korea, ◆-Afghanistan, ❖-Variety.

Our study has shown that the RAPD is very efficient in the production of DNA polymorphism in *Vigna mungo*, whereas intra-specific diversity through seed proteins have been reported low (Ghafoor & Arshad, 2008b; Rao *et al*., 1992) except for other crops where seed proteins have been effectively utilized (Siddiqui *et al*., 2010; Kumamaru *et al*., 2010). The methodology is relatively simple to perform, rapid and amenable to automation. The protocol is ready to be used in breeding, for registration and control of the distribution of commercial cultivars, the control of seed purity and for the cataloguing of germplasm collection as well. In future prospects, the results of this study can lead to a creation of group/variety-specific probes, using PCR primers (Paran & Michelmore, 1993). Cluster analysis based on agriculturally important traits revealed that high agronomic scores may be 0due to selection pressure for yield potential and other related characters. This revealed that only a portion of genetic diversity has been exploited, and it is suggested to broaden the genetic base of cultivated blackgram involving diverse parents in breeding program. Cluster analysis shows that many genotypes from same origins were grouped together as observed in cluster and PCA for quantitative traits and RAPD markers, thus technique could be used for identification of cultivars through RAPD fingerprinting. Grouping of germplasm originated from Pakistan in different clusters may be because of frequent exchange of germplasm by the breeders. Integration of more molecular markers and morphological traits to the blackgram linkage map will enhance its usefulness for marker-assisted selection. The genetic linkage map of blackgram developed here is currently being used to analyze QTL for seed weight, yield related and developmental traits. These results are in accordance to the studies Priya Srivastava *et al*., (2011).

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