



Genetic diversity analysis of blackgram [*Vigna mungo* (L.) Hepper] using morphological and molecular markers

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Abstract

A total of 30 blackgram [*Vigna mungo* (L.) Hepper] genotypes including five locally adapted varieties, twenty three advanced breeding lines and two local germplasm were evaluated for genetic diversity and relatedness with 21 morphological markers and 12 SSR markers. Genotypes viz., Uttara-IVT, Pant U 31-AVT, KUG 503, VBG 09-005 and NDU 11-202 were identified as the early flowering while DBGV 5, Vijay and WBB-1 were identified as tall having plant height more than 60 cm. Seed size (wt. of 100 seeds) ranged from 1.36 g in WBB-1 to 6.30 in COBG 759. The number of alleles per SSR marker varied from 3 to 5 per locus. Polymorphic information content values (PIC) ranged from 0.221 to 0.682 per locus. Variability among groups ($FIS=0.981$) and variability within individuals ($FIT=0.986$) was low. The most informative loci were CEDG 180, CEDG 139 and CEDG 279 with 5 alleles, CEDG 006, CEDG 143 and CEDG 056 with 4 alleles and CEDG 282, CEDG 204, CEDG 118, CEDG 068, CEDG 008, CEDG 043 with 3 alleles each. The minimum and maximum molecular genetic distances were found to be 0.00 (PU 08-2 with AKU 10-1) and 1.00 (NDU 11-202 with AKU 10-1, SB 44-4) respectively. Genotypes WBB-1, VBG 09-005 and IPU 10-17 showed high level of genetic diversity. Blackgram improvement through hybridization by utilizing diverse genotypes is suggested for breeding suitable genotypes for North Eastern region.

Key words: Fixation index, Genetic diversity, genetic distances, SSR

I. INTRODUCTION

Blackgram [*Vigna mungo* (L.) Hepper], popularly known as urdbean or mash, is a self-pollinating grain legume domesticated from *V. mungo* var. *silvestris* [1]. It is cultivated in many tropical and subtropical countries of the world. Blackgram is widely cultivated in the Indian subcontinent and to a lesser extent in Thailand, Australia, and other Asian and South Pacific countries [2]. It is cultivated as fallow crop after rice cultivation in India. It is grown in various agro-ecological conditions and cropping systems with diverse agricultural practices [3]. In various parts of India, particularly the hilly regions, a number of traditional landraces of blackgram are still being cultivated as inter crop between rice, sugarcane, cotton, groundnut and sorghum cultivating seasons. These landraces possess unique traits (e.g. disease tolerance, abiotic stress tolerance, pest tolerance), which have been identified by the farmers. India is the largest producer and consumer of blackgram in the world.

In recent years, there has been a significant decline in the pulse production in India. Lack of suitable varieties and genotypes with adaptation to local conditions is among the factors affecting the production. The major constraints in achieving higher yield of this crop are lack of genetic variability, absence of suitable ideotypes for different cropping systems, poor harvest index and susceptibility to diseases. Research on this species has lagged behind that of cereals and other legumes. Genetic diversity is a prerequisite for increasing yields and for stabilizing production in the face of disease epidemic and fluctuation in environmental conditions. Therefore, improvement of this crop is needed through

utilization of available genetic diversity. Genetic diversity is an important factor and also a prerequisite in any hybridization programme. Evaluation of genetic diversity would promote the efficient use of genetic variations in the breeding programme [4]. The accurate estimation of genetic diversity can be invaluable in the selection of diverse parental combinations to generate segregating progenies with maximum genetic variability. The study of genetic diversity can be particularly useful for precise identification of pure lines or cultivars with respect to plant varietal protection and germplasm maintenance by removing the duplicity and misidentity in the core accessions. Furthermore, monitoring the genetic variability within the gene pool of elite breeding material could make crop improvement more efficient by the directed accumulation of favoured alleles. Hence, the present study was carried out to assess the level of genetic diversity and relationship among the blackgram genotypes grown and evaluated in the North Eastern region for their future utilization in plant breeding.

II. MATERIALS AND METHODS

The genetically pure seed material of 30 blackgram genotypes including 21 advanced breeding lines, 5 locally available materials and 4 adapted genotypes were taken to evaluate genetic diversity and relatedness. All the advanced breeding lines meant for evaluation in the North Eastern Hill region were collected from Indian Institute of Pulse Research (IIPR), Kanpur through AICRP's (All India Coordinated Research Project) Centre, ICAR-NEH region. The adapted varieties of this region and local cultivars were collected from the Directorate of Research, Central Agricultural University, Imphal. The details of the genotypes along with their pedigree and origin/source are given in Table 1.

Morphological marker analysis

All the 30 accessions for morphological data analysis were sown in the college research farm, College of Post Graduate Studies (Central Agricultural University), Umiam, Meghalaya. Twenty-one morphological traits and scores were recorded as per the National Test Guidelines for the Conduct of Tests for Distinctness, Uniformity and Stability of twenty-one traits of black gram [*Vigna mungo* (L.) Hepper]. Five random plants from each plot were used to record the data on qualitative and quantitative characters. For analysis of morphological data, each character is treated to be a locus and the corresponding score for the phenotype is considered as an allele. Then, the data is used as an input to find out the Rogers' genetic distance and constructing corresponding tree using UPGMA method with Power Marker Software Version 3.25 [5].

SSR analysis

DNA extraction/PCR amplification and electrophoresis:

Total genomic DNA was extracted using the CTAB (Cetyl trimethylammonium bromide) method [6] with minor modifications. The quality of DNA extracted was visualized on 0.8 % agarose gel. For SSR analysis, 20 SSR markers were randomly selected with a probable total coverage of the black gram chromosomes.. Out of the 20 amplified primers, eight were removed because of their monomorphic nature. The PCR reactions were performed in a 10 µl final volume reactions containing 3 µl template DNA, including 0.08 µl Taq polymerase (5 units/µl, Fermentas), 1 µl of 10 X PCR buffer (Fermentas), 0.5 µl of DMSO, 0.3 µl of d NTP, 0.3 µl of the forward and reverse oligonucleotide primers (10 µM). The PCR conditions were: 95°C for 3 min followed by 34 cycles of 30 seconds at 94°C, 30 seconds at 55-60°C and 45 seconds at 72°C. Final elongation step was at 72°C for 3 min. The samples of PCR amplification were stored at 4°C before loading. The amplified products were electrophoretically separated at 90 V in a 2.5-3% agarose gels (Sigma, UltraPure Agarose 1000) for 2 h

along with the 100 bp Plus Ladder (Fermentas, Generuler), stained with ethidium bromide, visualized by UV light of Biorad Gel Documentation System and documented thereafter.

Genotype score for SSR data:

For each SSR marker and each sample, fragment sizes were visualized by comparison with the 100 bp Plus Ladder (Generuler, Fermentas) and genotype scoring was carried out manually as **a/a** for the single (homozygous) band and **b/b, c/c, d/d** for the next higher bands. Also the alleles were assigned band size relative to the molecular size ladder. Genotype data in numerical digit of the respective band size was archived in Excel tables for further analysis with with the software Power Marker Version 3.25 [5] and Arlequin Version3.5.1.2 [7].

III. RESULTS AND DISCUSSION

Morphological Data:

According to the morphological data collected on the different traits of the 30 genotypes of blackgram under the environmental conditions in Meghalaya, corresponding scores were given based on the DUS scores. Hypocotyl anthocyanin colouration was observed in 16 genotypes and it was absent in the rest of genotypes studied. For the plant growth habit, 19 genotypes were of spreading type and 11 genotypes were of semi-erect growing habit. Seventeen genotypes were having indeterminate growth habit and 13 genotypes determinate habit. The stem pubescence was present in all the genotypes. The genotypes which show early flowering were Uttara (IVT), KUG 503, VBG 09-005, NDU 11-202 (37 days), Vijay (39 days) and wild blackgram (WBB-1) showed late flowering (62days). The terminal leaflet shape was of lanceolate type in 7 genotypes, deltoid type in 9 genotypes, ovate type in 8 genotypes and cuneate type in 6 genotypes. The foliage colour of 13 genotypes was green and 17 genotypes had dark green foliage colour. For leaf vein colour, 19 genotypes had green colour and 11 genotypes had purple colour. All the genotypes had leaf pubescence. The petiole colour of 27 genotypes was green and 3 genotypes had green with purple splashes (RVSU 60, KPU 01-10 and VBG 09-005). The intensity of the colour of premature pods was observed to be green in 28 genotypes, dark green in 2 genotypes (IPU 10-17, VBG 09-005) and the colour of mature pods was brown in all the genotypes. The pod pubescence was present in 26 genotypes and absent in 4 genotypes (AKU 07-1, IGKU 03-16, DBGV 5, Vijay). The peduncle was of medium length for 19 genotypes (5-19 cm) and 11 genotypes had long peduncle length (>10 cm). For pod length, 18 genotypes were medium (5-7 cm), 11 genotypes were short (<5 cm) and 1 genotype were long (WBB 1- 8.7cm). The plant height of 20 genotypes was short (<45 cm), 7 genotypes were of medium height (45-60 cm) and 3 genotypes were long (DBGV 5, Vijay, WBB 1). Considerable variation existed for seed colour, seed shape and seed size. Nine genotypes had mottled seed colour, 18 genotypes had black and 3 had brown seed colour (IPU 10-17, KU 323 and LBBR 1). The seed lustre was dull in 26 genotypes and shiny in 4 genotypes (IPU 10-17, Bidhan Kalai 1, LBBR1 and WBB1).

Table 1. Details of the 30 blackgram genotypes including name of the genotype, their pedigree and origin/source

Sl.No.	Code	Genotype	Pedigree	Origin/Source
A.	Advanced breeding lines			
1.	KU-11-603	AKU 07-1	Mutant of TAU-1	PDKV, Akola
2.	KU-11-605	IGKU 03-16	PDU 1 x TU 94-2	IGKV, Raipur

3.	KU-11-606	PU 08-2	UPU 99-10 x KU 96-3	GBPUAT, Pantnagar
4.	KU-11-651	NDU 11-201	NDU Sel.17 x NDU 1	NDUA & T, Faizabad
5.	KU-11-652	AKU 10-1	TAU 1 x AKU 18-1	PDKV, Akola
6.	KU-11-655	KUG 503	KUG 15-1 x RBL-1	PAU, Ludhiana
7.	KU-11-656	DBGV 5	TAU 1 x LBG-20	UAS, Dharwad
8.	KU-11-659	SB 44-4	NDU 94-1 x NDU 95-8	AAU, Shillongani
9.	KU-11-660	IPU 10-17	UH 85-5 x PDU 103	IIPR, Kanpur
10.	KU-11-661	Vijay	Selection from TAU 1	Akola (MSSCL)
11.	KU-11-662	UH 08-05	DU 04 x UK 17	CCSHAU, Hisar
12.	KU-11-664	TU-136	Mutant of TU 94-2	BARC, Mumbai
13.	KU-11-667	ACM 05007	Co 5 x VBN(Bg) 4	Madurai
14.	KU-11-668	RVSU 60	OBG 27 x RBU 38	Sehore
15.	KU-11-669	KPU 01-10	RBU 38 x M 1-1	ARS, Banaswara
16.	KU-11-672	PU 09-22	UPU 97-10 x DPU 88-31	GBPUAT, Pantnagar
17.	KU-11-674	KU 323	T 65 x 766819	CSAU, Kanpur
18.	KU-11-677	Bidhan Kalai-1	Mutant of B-76	Mohanpur (BCKV)
19.	KU-11-679	COBG 759	VBN(Bg) 5 x <i>V. mungo var. silvestris</i>	TNAU, Coimbatore
20.	KU-11-681	KKB 05011	COBG 643 x VBN 3	Killikulam
21.	KU-11-682	VBG 09-005	VBN 3 x VBN 04-008	NPRC, Pudukkottai
22.	KU-11-683	NDU 11-202		
23.	Maintained at CAU, Imphal	COBG 653	DU 2 x VB 20	TNAU, Coimbatore
B.	Locally adapted varieties			
1.	KU-11-602	Uttara (IVT)	NP 19 x T9	IIPR, Kanpur
2.	KU-11-670	Uttara (AVT)	NP 19 x T9	IIPR, Kanpur
3.	KU-11-604	Pant-U 31 (IVT)	UPU 97-10 x DPU 88-31	GBPUAT, Pantnagar
4.	KU-11-654	Pant-U 31(AVT)	UPU 97-10 x DPU 88-31	GBPUAT, Pantnagar
5.	Maintained at CAU, Imphal	Pant U 31	UPU 97-10 x DPU 88-31	GBPUAT, Pantnagar
C.	Local Germplasm of Blackgram			
1.	LBBR-1	Local blackgram (Brown)	Local germplasm	CAU, Imphal
2.	WBB-1	Wild blackgram (Black)	Wild germplasm	CAU, Imphal

The seeds collected were of drum shaped for 13 genotypes and oval shaped for 17 genotypes. The weight of 100 seeds was of medium size for 19 genotypes with weight ranging from 3-5 g, large size for 10 genotypes with weight ranging from 5-6.30 g and Wild black gram had small size seeds with a weight of 1.36 g.

Morphological data showed that there was high variation for most of the morphological characteristics recorded, namely plant height, flowering time, seed size, plant growth habit, stem colour,

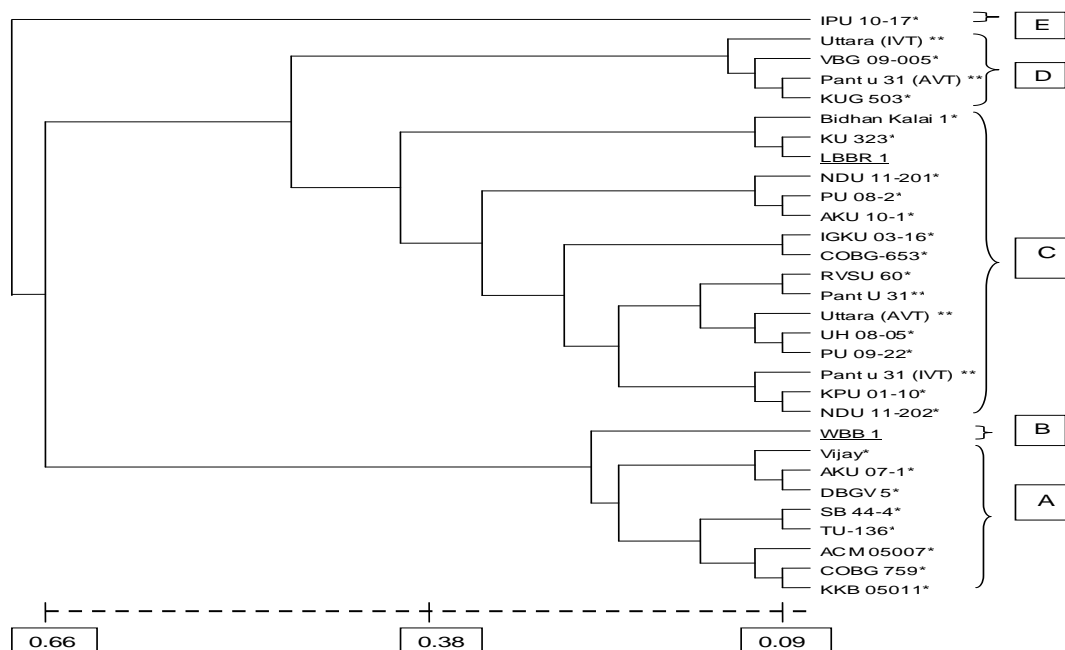
leaflet shape, peduncle length, pod length and seed shape. This high variation among the individual genotypes might be due to the use of genotypes in the study which were developed by different breeding institutes using different pedigree, breeding methods and also due to the inclusion of local landraces. 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 [8].

Morphological marker-based genetic distance and UPGMA cluster analysis

The minimum genetic distance based on morphological markers was 0.0952 which were found in TU-136 with SB 44-4 and PU 09-22 with UH 08-05 showing their close relatedness to each other. The maximum genetic distance observed was 0.67 in the genotype Uttara (AVT) with AKU 07-1, Vijay with IPU 10-17, WBB 1 with Pant U 31 (AVT) and WBB 1 with VBG 09-005. The average genetic distance based on morphological data was 0.38.

Five major clusters were obtained by truncating the dendrogram at the distance value of 0.30. On examining the dendrogram, it was found that 7 genotypes of the advanced breeding lines were grouped together in one extreme group labelled as A with Vijay and AKU 07-1 more closely related. WBB 1 formed a separate group in B. In group C, 3 locally adapted varieties (Pant U 31-IVT, Uttara (AVT), Pant U 31), 1 local germplasm (LBBR-1) and the 12 advanced breeding lines formed the cluster. Group D was formed by 2 locally adapted varieties (Uttara IVT, Pant U 31- AVT) and 2 advanced breeding lines (KUG 503, VBG 09-005). IPU10-17 formed a separate group from the rest of the genotypes in E.

The morphological-marker-based UPGMA cluster analysis demonstrated that all the advanced breeding lines and locally adapted varieties were somehow identical to the local germplasm in their morphology. The Dissimilarity coefficient which was evaluated on the basis of Roger’s distance [9] and expressed in UPGMA Dendrogram and unrooted tree, identified 5 major clusters, showing that varieties were highly similar within the clusters except for IPU 10-17 and WBB-1 which formed a separate group (Fig.1A).



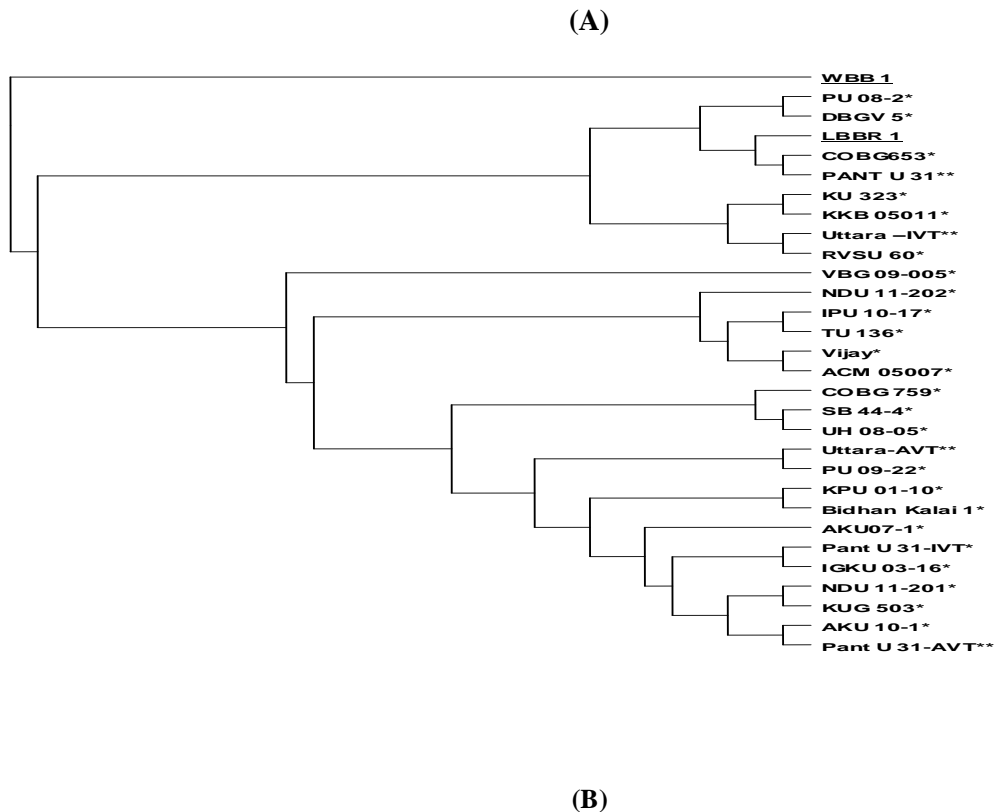


Fig. 1. Dendrogram based on Rogers' genetic distance (Rogers, 1972) among 30 black gram genotypes using (A) 21 morphological markers and (B) 12 SSR markers. Note: (*) = advanced breeding lines, (**) = locally adapted varieties, underlined = local germplasm.

Molecular variation

Thirty blackgram accessions (Table 1) were analyzed by using 12 polymorphic primers. Fragment size ranged from 130 to 300 base pairs. The total number of alleles observed for the 12 SSR loci was 45 and number of alleles per locus varied from 3 to 5 with an average number of 3.750 alleles per locus (Table 2). The frequency of the major allele for the locus CEDG 043 was high (0.8667) due to frequent occurrence of only one of the alleles (major allele) and minimum for the locus CEDG 180 (0.3667) on account of random occurrence of the alleles across the genotypes. Heterozygosity was observed in the loci CEDG 139 with 3% with the overall mean of 0.2% which was found to be very low. Polymorphic information content values (PIC) ranged from 0.2211 to 0.6827 per locus with an average 0.4383. Fixation index ranged from 0.9550 to 1.000 with an average of 0.9944 which was found to be high. The number of alleles observed suggested that the most informative loci were CEDG 180, CEDG 139 and CEDG 279 with 5 alleles, CEDG 006, CEDG 143 and CEDG 056 with 4 alleles and CEDG 282, CEDG 204, CEDG 118, CEDG 068, CEDG 008 and CEDG 043 with 3 alleles each. Expected heterozygosity was the highest for CEDG 180 and the lowest for CEDG 043. The allele frequencies were found to be highest for the 190 bp allele of the locus CEDG 043, the 180 bp allele of the locus CEDG 204 and the 120 bp allele of the locus CEDG 068. The allele frequencies were found to be lowest for the 220 bp allele of the locus CEDG 139, the 200 bp allele of the locus CEDG 006, the 120 bp allele of the locus CEDG 043 and the 130 bp allele of the locus CEDG 068. The variances accounted by the alleles were high for the 190 bp allele of the locus CEDG 056 and 185 bp alleles of the locus CEDG 279 (Fig.2) and low for the 155 bp allele of the locus CEDG 143, 190 bp allele of the locus CEDG 204, 130 bp allele of the locus CEDG 068 and the 200 bp alleles of CEDG 043.

Molecular marker (SSR)-based genetic distance and UPGMA cluster analysis

The minimum and maximum genetic distances were found to be 0.00 (PU 08-2 with AKU 10-1) and 1.00, respectively with an average of 0.5. The result of UPGMA cluster analysis based on Rogers' genetic distance calculated from the molecular marker data is presented in Fig. 1(B). The UPGMA cluster analysis revealed four major groups. From the dendrogram obtained, it was observed that the advanced breeding lines were clustered in group I and III. The locally adapted varieties were found in different major groups. The local cultivars were clustered in group III and group IV. The results revealed that the frequency of the major allele for the locus CEDG 043 was high due to frequent occurrence of only one of the alleles i.e. the major allele and minimum for the locus CEDG 180 on account of random occurrence of its alleles across the genotypes. Heterozygosity was observed in the loci CEDG 139 with 3% with the overall mean of 0.2%. Polymorphic information content values (PIC) ranged from 0.2211 to 0.6827 per locus with an average 0.4383 which was found to be relatively lower than the values recorded by Srivastava *et al.*[10] but similar to the values recorded by Tantasawat *et al.*[11]. The total and the average number of alleles obtained in the present study was low as compared to the results obtained by Tantasawat *et al.*[11] Deepak *et al.* [12] and Kanimozhi *et al.*[13], Souframanien and Gopalakrishna [14], Ajibade *et al.* [15] due to the relatively small population studied and few polymorphic markers. In the work carried out by Kanimozhi *et al.*[13], the number of alleles produced by different ISSR primers ranged from eight to 17 with an average of 11.5 per primer and the level of polymorphism was found to be 82.05 percent. High fixation index in most of the loci was accounted due to self pollination in black gram genotypes with minimal number of heterozygous loci. High fixation index was found to correlate with low variance.

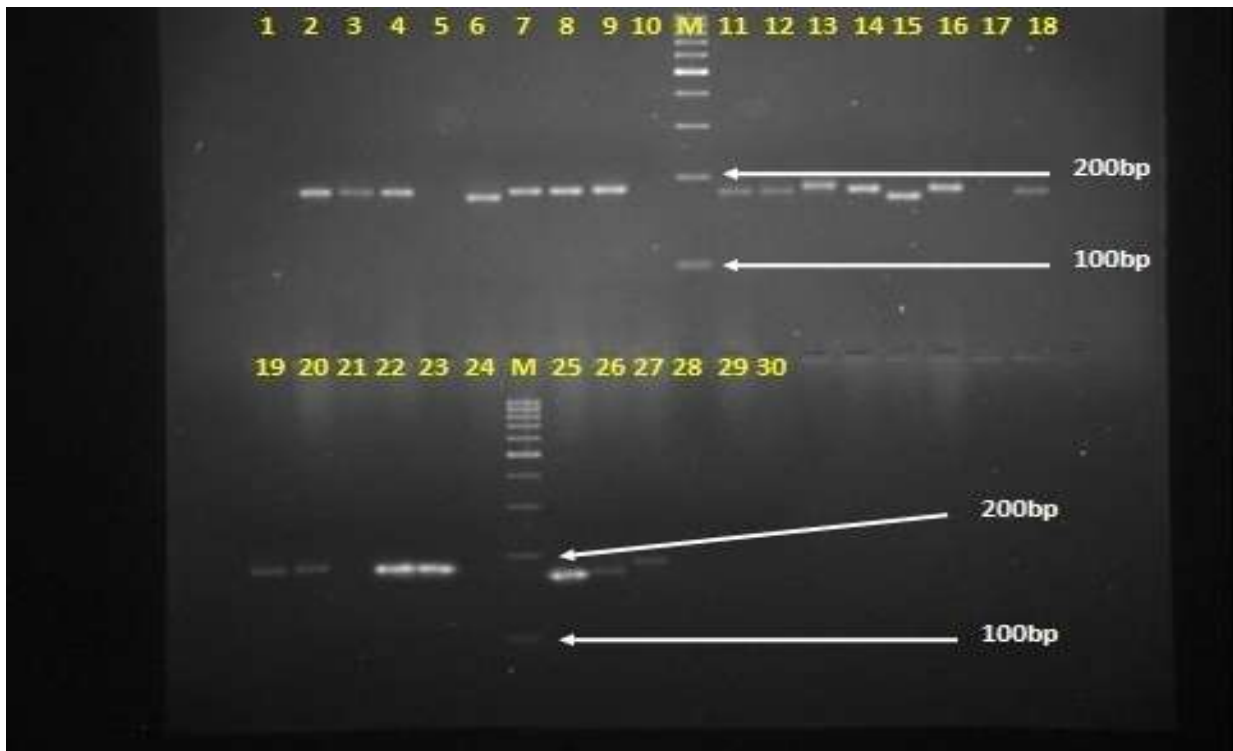


Figure 2: SSR pattern for the locus CEDG 279, M=molecular size ladder 100 base pairs. Serial number of the genotypes corresponds to Table1.

Table 2: Molecular diversity of the 12 SSR loci across 30 blackgram genotypes

Marker	Allele No.	Gene Diversity	Heterozygosity	PIC	f
CEDG056	4.0000	0.5844	0.0000	0.4999	1.0000
CEDG282	3.0000	0.4644	0.0000	0.4188	1.0000
CEDG143	4.0000	0.3444	0.0000	0.3244	1.0000
CEDG180	5.0000	0.7289	0.0000	0.6827	1.0000
CEDG204	3.0000	0.2867	0.0000	0.2604	1.0000
CEDG118	3.0000	0.5978	0.0000	0.5169	1.0000
CEDG139	5.0000	0.7161	0.0333	0.6702	0.9550
CEDG068	3.0000	0.2867	0.0000	0.2604	1.0000
CEDG008	3.0000	0.5311	0.0000	0.4745	1.0000
CEDG279	5.0000	0.6222	0.0000	0.5838	1.0000
CEDG043	3.0000	0.2378	0.0000	0.2211	1.0000
CEDG006	4.0000	0.3822	0.0000	0.3468	1.0000
Mean	3.8333	0.4819	0.0028	0.4383	0.9944
TOTAL	45.0000				

Where PIC= Polymorphism Information Content and f= fixation index

Analysis of molecular variance (AMOVA) was carried out to assess variability among, within groups and within individuals. The among-group variance component (Va), among individual within group variance component (Vb) and within individual variance component (Vc) were found to be 0.33379, 0.95971 and 0.01786, respectively. Percentage of variation contributed by Va, Vb and Vc were 25.45%, 73.18% and 1.36%, respectively. Among individuals within group variance was found to be high as compared to the among groups.

Correlation between morphological and molecular markers

Correspondence between the morphological marker-based distance matrix and the molecular marker-based distance matrix was tested with the Mantel Z statistic [16]. The significance of Z was determined by comparing the observed Z values with a critical Z value obtained by calculating Z for 1 matrix with 1000 permuted variants of the second matrix. All computations were done with the Power Marker Version 3.25 and the correlation was found to be 0.0120 at $p=0.4320$. The correlation was found to be very low, positive but significant at 5% level of probability.

Identification of diverse genotypes

The results of cluster analysis may be used to design a strategy to generate the genetic diversity in future varieties by crossing the local cultivars with the adapted ones. Another approach is to cross high yielding parents that possessed many random genetic differences which may increase the number of transgressive segregants. Morphological-based dendrogram revealed that genotypes IPU 10-17, VBG 09-005, Vijay and WBB 1 were diverse in their morphological characters. The diverse genotypes revealed from the molecular based dendrogram were WBB 1, VBG 09-005, LBBR-1, ACM 007, NDU 202 and RVSU 60. The 0.09 genetic distances as indicated between the paired genotypes, TU-136 and SB 44-4, PU 09-22 and UH 08-05 by morphological markers were further differentiated to a genetic distance of 0.41 and 0.33 respectively indicating robustness of molecular marker system. This study revealed that both the marker systems are important and supplemented each other as they have separate list of genotypes identified as diverse in which some genotypes are common.

On the basis of the present finding, it can be concluded that although only 30 genotypes were studied, estimated genetic variability among genotypes was high enough for creation of new favourable gene combinations. It is further suggested that the advance breeding materials of black gram from IIPR, Kanpur had sufficient genetic diversity to suit varied agro-ecological situations. Results indicated that the inter-crosses between already adapted varieties and advanced breeding lines as well as inclusion of valuable landraces into breeding programmes might prevent loss of diversity in the *Vigna mungo* gene pool. The diverse genotypes identified by the morphological and molecular analysis may be used in black gram improvement programmes.

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