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Molecular characterization using RAPD marker of Blackgram

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Abstract:

RAPD assay was conducted for molecular genetic analysis. Out of 13 random primers tested, finally three (S1027, S1239 and OPP13) were used. The primers yielded a total of 24 countable band of which 9 (37.5%) were considered as polymorphic. The highest number of bands (10) was generated by the primer S1239 whereas the lowest number of bands was (6) generated by the primer S1027. BARIMAS-1 showed the highest gene frequencies comparative to the other germplasms and BD10035 showed the lowest gene frequencies. The intra-germplasm

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similarity indices (Si) were found higher than the inter germplasm similarity indices (Sij). Among the three primers, OPP13 showed the highest and S1027 showed the lowestintra-germlpasm (100%) similarity indices. The highest Inter-germplasm similarity index (100%) was found between BD10035 vs. BD10042, BD10036 vs. BD10037 and BD10035 vs. BD10047. On the other hand, BARIMAS-2 vs. BD10039 showed the lowest inter-germplasm similarity index. The dendrogram had indicated segregation of ten germplasms of Blackgram into two main clusters. In cluster-I, BARIMAS-1 and BD10034 formed sub cluster-I. BD10039 and BD10047 formed sub cluster II. In cluster II, BARIMAS-2, BD10036, BD10037, and BD10033 formed sub cluster I. Again in sub cluster I BARIMAS-2 alone formed sub sub-cluster I, BD10036 and BD10037 formed sub sub-cluster II, and BD10033 alone formed sub sub-cluster III. BD10035, BD10042 formed sub cluster II. This study indicates the highest genetic variation between BD10036 Vs BD10037 and the lowest genetic variation between BARIMAS-2 Vs BD10039 that can be used for breeding programs to improve Blackgram varieties.

Key words: Black gram, random amplified polymorphic deoxyribonucleic acid, genetic diversity, similarity coefficient.

INTRODUCTION

Blackgram (*Vignamungo* L. Hepper, 2n=22) is a self pollinating and widely cultivated grain legume (Nag *et al*., 2006) belongs to the family Leguminosae, sub-family Papilionaceae. It is one of the most important pulse crops grown in Bangladesh. It contains approximately 25-28% protein, 4.5-5.5% ash, 0.5-1.5% oil, 3.5-4.5% fibre and 62-65% carbohydrate on dry weight basis (Kaul, 1982). Blackgram is one of the rich sources of vegetable protein and some essential mineral and vitamins for human body. It has the ability to fix atmospheric nitrogen. There are numerous techniques available for assessing the genetic variability and relatedness among crop germplasm. DNA based markers are effective and reliable tools for measuring genetic

diversity in crop germplasm and studying evolutionary relationship. Molecular genetics techniques using DNA polymorphism is increasingly used to characterize and identify a novel germplasm for uses in the crop breeding process (O' Neill *et al.*, 2003). The evaluation based on Random Amplified Polymorphic DNA (RAPD) profiles would be suitable for providing such information due to its high level of polymorphism of this technique. Morphological characters facilitate in the identification and selection for the desirable traits (Nisar*et al.*, 2008). In recent years, DNA fingerprinting system based on RAPD analysis have been increasingly utilized for detecting genetic polymorphisms in several plant genera. This availability of genetic variability for a given character is a prerequisite for its upgrading by systematic breeding program. The evaluation of the genetic diversity would promote the efficient use of genetic variation in the breeding program (Paterson *et al.,* 1991). The specific objective of the study was to investigate and compare genetic diversity among ten elite genotypes of known origin, using RAPD.

MATERIALS AND METHODS

Plant materials and DNA extraction

Ten blackgram genotypes in Table 1 were used as experimental materials among which BARIMASH-1 and BARIMASH-2 were collected from Pulse Research Division, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur and the rest of eight germplasms were collected from Plant Genetic Resource Centre (PGRC), Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. The genotypes used in the study are listed in Table1. Young and healthy leaves were pooled from 20 to 25 days old plant, washed and quickly frozen and powdered using liquid nitrogen. DNA was extracted using the method described by Dellaporta *et al.* (1983).

Table 1: List of black gram genotypes used in the present study

Sl. no.	Genotype	Sl. no.	Genotype
1	BARIMASH-1	**6**	BD-10036
2	BARIMASH-2	**7**	BD-10037
3	BD-10033	**8**	BD-10039
4	BD-10034	**9**	BD-10042
5	BD-10035	**10**	BD-10047

Confirmation of DNA preparation

Sometimes isolated genomic DNA contains a large amount of RNA and pigments which usually cause over estimation of DNA concentration on a spectrophotometer. Thus, the DNA samples were evaluated both quantitatively and qualitatively using 0.8% agarose gel.

Quantification of DNA concentration

Different DNA extraction methods provide DNA of widely different purity. Thus, it is necessary to optimize the amount of DNA used in the RAPD analysis to achieve reproducibility and also the strong signal. Below a certain critical concentration of genomic DNA, RAPD amplification is no longer reproducible (Williams *et al.,* 1993). Thus, it is essential to keep on above such critical concentration. It is best to do a series of RAPD reaction using a couple of primers and a set of serial dilutions of each genomic DNA to identify empirically the useful range of DNA concentration, for which reproducible RAPD patterns are obtained.

After confirmation, purity and concentration of genomic DNA was examined by calculating the ratio of the optical density measured at 260 nm using a spectrophotometer and stored in freezer. The test samples were prepared by taking 2µl of each sample in a cuvette containing 2 ml sterile distilled water. The sample was uniformly mixed, placed in spectrophotometer and the absorbance reading was taken at 260 nm. Then the cuvette was rinsed out with sterile water, stamped out on paper wipe, and absorbance reading for each

sample was recorded in the same way. The results of the DNA concentration were obtained in µg/µl, this results containing a fraction. So, to avoid fraction it was converted in ng/µl (1 µg = 10^{-3}ng). Therefore, the result was multiplied with 1000.

Amplification of RAPD markers by polymerase chain reaction (PCR)

The RAPD analysis was performed at the USDA Biotech Lab, Department of Biotechnology, Faculty of Agriculture, BAU, Mymensingh. To perform amplification of RAPD, a single oligonucleotide of arbitrary DNA sequence is mixed with genomic DNA in the presence of a thermo stable.

DNA polymerase and suitable buffer, and then subjected to temperature cycling conditions typical to the polymerase chain reaction (PCR). The reaction involves repeated cycles, each consisting of a denaturation, a primer annealing and an elongation step. In the first step, the DNA is made single stranded by raising the temperature to 94°C (denaturation).

In the second step, lowering of the temperature to about an optimal annealing temperature, the primer binds to their target sequences on the template DNA (annealing step). In the third cycle, temperature is chosen as where the activity of the thermo stable TaqDNA polymerase is optimal, i.e., usually 72°C. The polymerase then extends the 3' ends of the DNA primer hybrids towards the other primer-binding site. Since this happens at both primer-annealing sites on both the DNA strands, the target fragment is completely replicated. Repeating these three cycles 40 to 50 times results in the exponential amplification of the target between the 5' ends of the two primer binding sites. Amplification products are separated by agarose gel electrophoreses and visualized by ethidium bromide staining.

Primer selection

Initially, thirteen primers (Table 2) of random sequence were screened on a sub-sample of two randomly chosen individuals from two different varieties to evaluate their suitability for amplifying Blackgram RAPDs that could be scored accurately. Primers were evaluated based on intensity of bands, consistency within individual, presence of smearing, and potential for population discrimination. A final subset of three primers out of thirteen exhibiting good quality banding patterns were selected for analysis of the whole sample set of the ten germplasms of Black gram

Table 2 Parameters of the random primers used in the present study for screening

Primer	Sequence	GC content (%)
S1027*	ACGAGCATGG	60
S1184	GACGGCTATC	60
S1234	TCGCAGCGTT	60
S1239*	TGACAGCCCC	70
S1265	GAGCTACCGT	60
S1320	CTACGATGCC	60
S1358	ACCCCAACCA	60
OPA02	TGCCGAGCTG	70
OPA10	GTGATCGCAG	60
PAB04	GGCACGCGTT	70
OPAB09	GGGCGACTAC	70
OPP13*	GGAGTGCCTC	70
OPW19	CAAAGCGCTC	60

* Selected for RAPD analysis for all samples of the ten germplasm

PCR amplification and agarose gel electrophoresis

The amplification conditions were based on Williams *et al.* (1990) with some modification. PCR reactions were performed on each DNA sample in a 10 µ1 reaction mix containing the reagents like 10x Ampli*Taq* polymerase buffer (32µl), dNTPs (32 µl), primers (64µl), $MgCl_2$ (19.2µl), template DNA (32 µl), Ampli*Taq* DNA polymerase (6.4 µl), and ddH_2O (134.4µl)

During the experiment, PCR buffer, dNTPs, primer, and DNA samples solutions were thawed from frozen stocks, mixed by vortexing and kept on ice. DNA template was pipetted first into PCR tubes compatible with the thermocycler used. A pre-mix was then prepared in the course of the following order: reaction buffer, dNTPs, DNA template, and sterile distilled water. *Taq*polymerase enzyme was then added to the pre-mix. The pre-mix was then mixed up well and aliquoted into the tubes that already contain primer. The tubes were then sealed and placed in a thermocycle and the cycling was started immediately. DNA amplification was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf). The reaction mix was preheated at 94°C for 3 minutes followed 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 36°C and elongation or extension at 72°C for 2 minutes. After the last cycle, a final step of 7 minutes at 72°C was added to allow complete extension of all amplified fragments. After completion of cycling program, reactions were held at 4°C. The amplified product from each sample was separated electrophoretically on 1.4% agarose gel contain ethidium bromide in 1X TBE buffer at 120 V for 1½ hrs. Molecular weight marker DNA (1kp &20kp ladder) was eletrophoresed alongside the RAPD reactions. DNA bands were observed on UV-translluminator and photographed by a Gel Documentation System.

Data Analysis

RAPD markers were scored visually of their presence (1) or absence (0), separately for each germplasm of Blackgram and each primer. For more accuracy, two independent persons performed band scoring. Bands not identified by the two readers were considered as non-scorable. The scores obtained using all primers in the RAPD analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, Nei's (1973) gene diversity, population

differentiation (G_{ST}), gene flow (N_m), gene distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Mean) dendrogram among populations using POPGENE (version 1.31) (Yeh*et al.*, 1999) computer program.

Estimation of gene frequencies of RAPD loci was based on the assumption of a two allele system. Of the two alleles, only one is capable of amplification of a RAPD band by primer annealing at an unknown genomic position (locus). The other is the 'null' allele incapable of amplification, mainly because of loss of the primer annealing site by mutation. The two allele assumption is in most cases acceptable, because co-dominant loci showing band shifts are few (Elo *et al.*, 1997; Welsh and McClelland, 1990). In this system, only a null homozygote is detectable as negative for the RAPD-band of interest. Under the assumption of Hardy-Weinberg equilibrium, the null allele frequency (q) may be (N/n)1/2, where N and n are the number of band negative individuals observed and the sample size, respectively. The frequency of the two alleles (P) is 1-q. The assumption of the two allele system enables us to calculate the Nei's genetic distance (Nei's, 1972) from the RAPD pattern. The similarity index values (SI) between the RAPD profiles of any two individuals on the same gel were calculated from RAPD markers according to the following formula:

$$\text{Similarity Index (SI)} = 2N_{AB}/ (N_A + N_B)$$

Where, N_{AB} is the number of RAPD bands shared by individual A and B and N_A and N_B, are the numbers of fragment scored for each individual respectively (Lynch, 1990). Within germplasm similarity [S_i] was calculated as the average of SI across all possible comparisons between individuals within a germplasm. Between germplasm similarity (S_{ij}) was calculated as the average similarity between randomly paired individuals from germplasm I and j (Lynch, 1998).

RESULTS AND DISCUSSION

Primer selection and RAPD patterns

Among the thirteen primer tested, tree primer (S1027, S1239 and OPP13) produced comparatively higher number of high intensity bands with minimal smearing. These three primers produced a total of 24 band of which 9(37.5%) was considered as polymorphic (Table 11). The banding patterns of different germplasms using primers S1027, S1239 and OPP13 were showed in figure 1, 2 and 3, respectively. The maximum number of bands (10) was produced by the primer S1239 followed by OPP13 (8), whereas the least number (6) was generated by primer S1027. Srivastava *et al.* (2011) observed 3 to 14 bands and the average number of markers produced per primer was 8.6. Shafique *et al.* (2011) revealed 1-5 amplified fragments through RAPD marker. On the other hand, the primer S1027 amplified higher percentage of polymorphic bands (16.67%), while the primer S1239 generated the least (8.33%) polymorphic bands. The average scorable and polymorphic bands produced by the three primers were 9, and 3, respectively. Weak bands results from low homology between the primer and the pairing site on the DNA strand (Thormann *et al.*, 1994).

Table 3. RAPD primers with corresponding bands scored together with polymorphic bands observed in ten Blackgram germplasms

Primer codes	Sequences (5'-3')	Total no. of bands scored	No. of Polymorphic bands	% of polym-orphic band
S1027	ACGAGCATGG	6	4	66.67
S1239	TGACAGCCCC	10	2	20.00
OPP13	GGAGTGCCTC	8	3	37.50
Total		24	9	37.50
Avg.		8	3	37.50

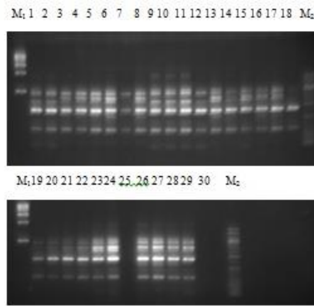

Fig. 1. RAPD profiles of ten germplasms of Blackgram using S1027 primer.

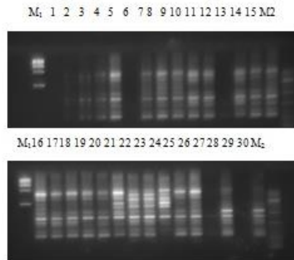

Fig. 2. RAPD profiles of ten germplasms of Blackgram using S1239 primer.

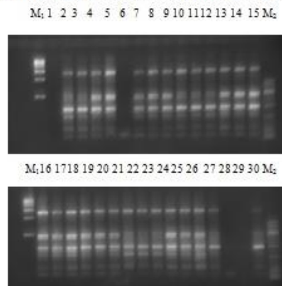

Fig. 3. RAPD profiles of ten germplasms of Blackgram using OPP13 primer

Frequency of polymorphic loci

The DNA polymorphisms were detected according to the presence and absence of bands. Absence of bands may be caused by failure of primers to anneal a site in some individuals due to nucleotide sequence differences or by insertions or deletions in primer sites (Clark and Lanigan, 1993). Frequencies of polymorphic RAPD markers are shown in Table 12. According to the results in Table 12, BARIMASH-1 showed highest gene frequencies comparative to the other germplasms and BD10035 showed less gene frequencies. The ability to resolve genetic variation may be more directly related to the number of

polymorphism detected by the marker techniques and the percentage of polymorphic RAPDs. However, it does not correlate with the influence of rare and common alleles on the genetic diversity as a fragment of the lowest frequency has the same importance as a fragment with the highest frequency across the genome (Welsh and McClelland, 1990; Ehlers and Hall, 1997).

Intra-germplasm similarity indices (Si)

The intra-germplasm similarity indices (Si) were found higher (ranged from 94.44-100% with an average of 98.26 %) than the inter germplasm similarity indices (Sij) ranging from 86.81-100% with an average of 93.39%. This implies that individuals within each germplasms are genetically more similar into each other, as it expected to be, than to individuals from all other germplasms. Highest (100%) intra germplasm similarity indices were found in BD-10035, BD-10036, BD-10037, BD-10039, BD-10047. On the other hand, BARIMASH-1 (94.44%) showed the lowest Intra-germplasm similarity indices. Among the three primers, OPP13 showed highest intra-germlpasm (100%) similarity indices while S1027 generated lowest intra-germplasm similarity indices (95.52%).

Inter-germplasm similarity indices (Sij)

Inter-germplasm means of the pair-wise similarity indices (Sij) ranged from 86.81% to 100%. The highest Inter-germplasm similarity index (100%) was found between BD10035 vs. BD10042, BD10036 vs. BD10037 and BD10035 vs. BINADHAN-7 (Table 13.B). So the genetic distance between these variety pair was lower. On the other hand, BARIMASH-2 vs. BD10039 showed the lowest inter-germplasm similarity index (86.81%) and also BD10037 vs. BD10039 and BD10036 vs BD10039 (88.03%), BARIMASH-1 vs. BD10035 (88.57%) and BARIMASH-1 vs. BD10042 (88.57%) were found lower. Thus a greater genetic distance between these germplasm exists. Inter

germplasm similarity indices for three primers were 89.81%, 99.24% and 91.49% for S1027, S1239 and OPP13 respectively.

Gene diversity and frequency of polymorphic loci

Genetic diversity within the germplasm for three primers is given in Table 14. The overall Nei's (1973) gene diversity and Shannon's Information indices were 0.1113 and 0.1711, respectively. High value of gene diversity (0.0407) and Shannon's Information index (0.0568) were found in BD10034 and BD10039. The lowest level of gene diversity and Shannon's Information index were found 0.0203 and 0.0284 in BARIMASH-1.

Nei's (1972) genetic identity and genetic distance

The highest Nei's (1972) genetic distance (1.00) was observed in BD10035 Vs BD10042 and BD10035 Vs BD10035 germplasms pairs whereas, the lowest genetic distance (0.79) was estimated in BARIMASH-2 Vs BD10039 germplasms pair (Table 12). Furthermore, high level of genetic distance was found in BARIMASH-1 Vs BD10034 (0.97); BARIMASH-2 Vs BD10033 (0.97); BARIMASH-2 Vs BD10036 (0.99) also for BD10033 Vs BD10036 and BD10033 Vs BD10037 (0.99) germplasms pair. A wide range of genetic distance observed between PGRC germplasm indicates a large genetic diversity among them. Low level of genetic distance was observed in BD10033Vs BD10039 (0.80); BARIMASH-2 Vs BINADHAN-7(0.82) germplasms pair. It indicates less diversity among them.

Table 4. Genetic diversity for three primers in all cultivars

Locus	No. of polymorphic loci	Percentage	Nei's (1973) gene diversity	Shannon's Information index
BARIMASH-1	2	8.33	0.03 ±0.11	0.05 ±0.17
BARIMASH-2	1	4.17	0.02 ±0.10	0.03 ±0.14
BD10033	2	8.33	0.03	0.05

			±0.11	±0.17
BD10034	2	8.33	0.04 ±0.14	0.06 ±0.19
BD10035	0	0.0	0.0	0.0
BD10036	0	0.0	0.0	0.0
BD10037	0	0.0	0.0	0.0
BD10039	2	8.33	0.04 ±0.14	0.06 ±0.19
BD10042	0	0.0	0.0	0.0
BINADHAN-7	0	0.0	0.0	0.0
Overall	9	37.50	0.11 ±0.18	0.17 ±0.26

Dendrogram

A dendrogram based on Nei's (1972) genetic distance using UPGMA, indicates segregation of ten germplasm of Blackgram into two main clusters: BARIMASH-1, BD10034, BD10039 and BINADHAN-7 grouped in cluster-I while BARIMASH-2 BD10036, BD10037, BD10033, BD10035 and BD10042 in cluster-II (Fig. 4). In cluste-I, BARIMASH-1 and BD10034 formed sub cluster-I. They were similar at 96.61 % similarity indices. BD10039 and BINADHAN-7 formed sub cluster II. They were similar at 91.06 % similarity indices. In cluster II, BARIMASH-2, BD10036, BD10037, and BD10033 sub cluster I. Again in sub cluster I BARIMASH-2 alone formed sub sub-cluster I, BD10036 and BD10037 formed sub sub-cluster II, they were similar at 100 % similarity indices and BD10033 alone formed sub sub-cluster III. BD10035, BD10042 formed sub cluster II. They were similar at 100 % similarity indices. This study indicates the highest genetic variation between BD10036 Vs BD10037 and the lowest genetic variation between BARIMASH-2 Vs BD10039 that can be used for breeding programmes that aim to improve Blackgram varieties. The results also revealed that the genetic base among these Blackgram.

CONCLUSION

The RAPD technique was used for assessing genetic variation and relationship among 10 Blackgram genotypes. Thirteen decamer random primers were initially screened and finally 3 primers S1027, S1239, and OPP13 were selected for the analysis. RAPD makers were amplified by PCR technique, separated in 1.4% agarose gel electrophoretically, stained in ethidium bromide solution and finally visualized on uv trans-illuminator.

A total of 24 RAPD bands were scored of which 9 polymorphic amplification products were obtained by using these primers. The highest number of bands (10) was produced by the primer S1239 followed by OPP13 (8), whereas the least number of bands was (6) generated by the primer S1027. BARIMAS-1showed highest gene frequencies comparative to the other germplasms and BD10035 showed lowest gene frequencies.

The intra-germplasm similarity indices (Si) were found higher than the inter germplasm similarity indices (Sij). Among the three primers, OPP13 showed highest intra-germplasm (100%) similarity indices while S1027 generated lowest intra-germplasm similarity indices (95.52%). The highest Inter-germplasm similarity index (100%) was found between BD10035 vs. BD10042, BD10036 vs. BD10037 and BD10035 vs. BD10047. On the other hand, BARIMASH-2 vs. BD10039 showed the lowest inter-germplasm similarity index. BARIMAS-1showed the lowest Intra-germplasm similarity indices. Highest intra-germplasm similarity indices was found in BD10035, BD10036, BD10037, BD10039, BD10047.

High value of gene diversity (0.0407) and Shannon's Information index (0.0568) were found in BD10034 and BD10039. The lowest level of gene diversity and Shannon's Information index were found 0.0203 and 0.0284 in BARIMAS-1.

Following the Unweighted Pair Group Method of Arithmetic Means (UPGMA) the 10 genotypes of Blackgram were grouped into 2 main clusters namely Cluster I and Cluster II. BARIMAS-1, BD10034, BD10039 and BD10047grouped in cluster-I while BARIMAS-2, BD10036, BD10037, BD10033, BD10035 and BD10042 in cluster-II. In cluste-I, BARIMAS-1and BD10034 were formed sub cluster-I. BD10039 and BD10047 formed sub cluster II. In cluster II, BARIMAS-2, BD10036, BD10037, and BD10033 sub cluster I. Again in sub cluster I, BARIMASH-2 alone formed sub sub-cluster I, BD10036 and BD10037 formed sub sub-cluster II, and BD10033 alone formed sub sub-cluster III. BD10035, BD10042 formed sub cluster II.

Genotypic variations based on molecular characterization indicated that genotypes belonging to different clusters depend on their genetic components itself, but not at geographical origin at all. Therefore, it could be concluded that for further research programme, especially for hybridization, genotype could be selected from different clusters will be provided maximum heterosis regarding yield. In conclusion, the result of the present experiment revealed that the variability existed among the selected Blackgram genotypes was not so wide. This variability may be also used for selection of superior genotypes for commercial cultivation at farmer's level as well as for breeding new genotypes of Blackgram in our country.

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