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Molecular Assessment of Genetic Diversity and Relationship in Selected Mungbean Germplasms

[1]Saiful Islam Bhuyan, [2]Md. Sanower Hossain, [3]Mirza Mofazzal Islam, [4]Shamsun Nahar Begum, [2]Zannat Urbi and [1]Md. Sumon Hossain
[1]Department of Biotechnology, Faculty of Agriculture,
Bangladesh Agricultural University (BAU), Mymensingh-2202, Bangladesh
[2]Department of Biotechnology, Faculty of Science,
International Islamic University Malaysia, Kuantan-25200, Pahang, Malaysia
[3]Division of Biotechnology,
[4]Division of Plant Breeding, Bangladesh Institute of Nuclear Agriculture (BINA),
Bangladesh Agricultural University Campus, Mymensigh-2202, Bangladesh

Abstract: Mungbean is an important crop considering the nutritional supplementary of low fat, high fiber and protein but its production is very low compared to the daily requirements. Hence, the assessment of genetic diversity and relationships in the existing germplasms is a major concern for the development of high yielding variety of mungbean. In this study, Random Amplified Polymorphic DNA (RAPD) marker was used to analyse 7 exotic and 3 advance germplasms using 3 primers (OPA01, OPB06 and OPB07). On an average 6 amplified products/primer were formed with overall polymorphisms of 78.33%. The similarity coefficient was highest (0.93) between AVRDC-3 and AVRDC-4, indicating less divergence and was least (0.39) between AVRDC-5 and AVRDC-6, indicating more divergence. On the basis of UPGMA dandogram, genotypes AVRDC-5, AVRDC-6 and AVRDC-7 were found to be quite distinct and the simple matching coefficient varied from 0.1824 to 0.8109. These findings will be of significance in the development of intraspecies crossing variety of mungbean crop.

Key words: Genetic distance, legume, RAPD, *Vigna radiata*

INTRODUCTION

The third largest family of higher plants is legume that comprises more than 650 genera and 18,500 species. Mungbean (2n = 2x = 22), *Vigna radiata* (L.) Wilczek, legume belongs to the family Leguminosae and occupies pivotal position among the pulses grown in Bangladesh. It is originated from Southeast Asia and found in India, Pakistan, Bangladesh, Mayanmar, Thailand, Philippines, China and Indonesia. The production of pulses is very low (0.239 million metric ton) compared to the total requirement (2.54 million metric tons) in Bangladesh that is indicating around 90% more production required in order to fulfill pulse based nutrition including protein, vitamins and minerals (Bisht *et al*., 2010). The total requirement of pulses was calculated based on desirable dietary pattern of pulse (50 g/capita/day) and total population of Bangladesh (139,252,683) in 2011 (BBS, 2013; Nahar *et al*., 2013). The consumption of pulses in Bangladesh is only 14.68 g/capita/day (Nahar *et al*., 2013) resulting the people are facing cereal based nutritional crisis. Mungbean are primarily grown for the protein rich edible seeds as foods due to easy digestibility by human (Poehlman, 1991). The seeds, sprouts and young pods of mungbean are consumed widely as sources of protein (26%), amino acids, vitamins (3%) and minerals (4%) and plant parts are used as fodder and green manure (Khan, 1981). In addition it is a good complementary food for rice based diets due to low fat, high fiber and high protein especially high lysine, the first limiting amino acids, content (504 mg g^{-1}) (Chen *et al*., 1987; Saini *et al*., 2010). Therefore it can be an excellent source of supplement protein to overcome the protein deficiency in Bangladesh as well as other countries.

The mungbean is widely cultivated for being a short growth duration (70-110 days) crop (Lawn, 1983). It requires low water and fertile soil to grow, is cultivated in crop rotation practices and therefore important as a soil nitrogen fixer for maintaining soil fertility. In Bangladesh, the different varieties of mungbean are found with such

Corresponding Author: Md. Sanower Hossain, Department of Biotechnology, Faculty of Science,
International Islamic University Malaysia, Kuantan-25200, Pahang, Malaysia Tel: +6016-3615 687

low yield due to several reasons including biotic and abiotic constrains (Mondal, 2007). The varieties grown are genetically low yield potential and also susceptible to pests and diseases. The mungbean production is correlated with primary yield components and also with other morphological and physiological characters. Therefore, attempts must be made to increase the yield per unit area by applying improved technology and management practices. The hybridization technique commonly practices to improve the crop varieties by crossing the parents with maximum genetic divergence (Arunachalam, 1981). The gene pool of mungbean can also be used for development of promising or superior varieties (Ramanujam, 1978). Hence, the screening and characterization of genetic diversity would be crucial steps to find potential parental accessions in order to develop the improved varieties of mungbean.

The morphological traits, worked as ambiguous markers because of environmental influences, used traditionally to characterize and catalogue the germplasms. Protein and isozyme electrophoresis are also used with insufficient polymorphism among the closely related cultivars. However, the molecular markers are proven to be a powerful tool and used from last three decades to characterize germplasms. Among the several molecular markers, RFLP, Simple Sequence Repeats (SSR), RAPD (Karp *et al*., 1997) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995) are commonly used to evaluate the genetic diversity and phylogenetic relationships in plant genetic resources.

The PCR based molecular marker RAPD developed by Williams *et al*. (1990) is reliable, rapid, easier, free from environmental influences, not limited in number, random but wide coverage of genome, require low quantities of template DNA (usually 5-50 ng per reaction) and have a relatively higher level of genetic polymorphism within and among species and populations (Gustine and Huff, 1999; Newbury and Ford-Lloyd, 1993). The RAPD analysis has been applied to characterize germplasms in case of several crops including (Daivila *et al*., 1999), black gram (Arulbalachandran *et al*., 2009; Souframanien and Gopalakrishna, 2004; Srivastava *et al*., 2011), cluster bean (Pathak *et al*., 2010), cowpea (Ghalmi *et al*., 2010), pigeonpea (Choudhury *et al*., 2008), popcorn (Leal *et al*., 2010) and *Vigna* species (Dikshit *et al*., 2007; Undal *et al*., 2011), wheat (Cao *et al*., 1998; Fahima *et al*., 1999; Joshi and Nguyen, 1993; Vierling and Nguyen, 1992) and spice herb coriander (Pareek *et al*., 2011; Singh *et al*., 2012). In case of mungbean, several previous studies also have been conducted using RAPD profiles alone or combination with Inter Simple Sequence Repeat (ISSR) (Afzal *et al*., 2004; Betal *et al*., 2004; Bisht *et al*., 1998; Cattan-Toupance *et al*., 1998; Chattopadhyay *et al*., 2005; Datta *et al*., 2012; Dikshit *et al*., 2009; Freyre *et al*., 1996; Lakhanpaul *et al*., 2000; Lavanya *et al*., 2008; Saini *et al*., 2004, 2010; Santalla *et al*., 1998; Sonnante and Pignone, 2001; Taunk *et al*., 2012; Tosti and Negri, 2002; Xu *et al*., 2000; Xuzhen *et al*., 1996). Based on the earlier studies it can be concluded that RAPD markers were appropriate for gene mapping, as well as DNA fingerprinting and population genetic studies. Random Amplification of Polymorphic (RAPD) analysis can efficiently be used to reveal genetic relationships among different varieties of a species. To our knowledge, only one study (Afzal *et al*., 2004) was carried out in Taiwan using few selected varieties from Bangladesh, Asian Vegetable Research and Development Centre (AVRDC), India and Thailand to investigate the genetic diversity of mungbean using RAPD marker techniques and no other study carried out yet in Bangladesh even in other countries except very few selected Bangladeshi cultivars. Therefore, the aim of this study was to assess the genetic variability and phylogenetic relationships among ten germplasms of mungbean under cultivation in Bangladesh, based on RAPD markers which might facilitate the breeder to introduce agronomically important traits.

MATERIALS AND METHODS

Study materials: The plant materials (seeds) of the present study were collected from AVRDC (7 germplasms) and Bangladesh Institute of Nuclear Agriculture (BINA) (3 germplasms) (Table 1). All the collected seeds were round shaped and these seeds were sown and grown at the Laboratory's field, Division of Biotechnology, BINA. Young and fresh leaves were collected from 28 days old seedlings of each genotype, quickly washed with distilled water and dipped in ethanol for few second followed by

Table 1: Source of mungbean germplasms used for RAPD analysis

Source and remarks	Sample No.	Genotype
AVRDC		
Exotic mungbean lines	MB09	AVRDC-3
	MB10	AVRDC-4
	MB11	AVRDC-5
	MB12	AVRDC-6
	MB13	AVRDC-7
	MB15	AVRDC-9
	MB21	AVRDC-15
BINA		
Advanced mutant lines	MB22	MBM-390-394(1)
	MB23	MBM-657
	MB32	MBM-590-93

dried on blotting paper and finally stored at -80°C using zip lock bags until total genomic DNA was extracted.

Genomic DNA extraction and quantification: The total genomic DNA was extracted following the modified method of Saghai-Maroof *et al.* (1984). The frozen leaves (~0.7 g) were ground in liquid nitrogen to a fine powder using mortar and pestle. DNA extraction buffer containing 0.5 M Ethylene Diamine Tetra-acetic Acid (EDTA) (pH 8.0), 1 M tris-HCl at pH 8.0, 5 M NaCl) and 50 µL 20% SDS was added to the powder tissue (1 mL g^{-1} fresh weight). The equal volume (100 µL) of 5 M NaCl and Cetyl Trimethyl Ammonium Bromide (CTAB) were added to the suspension, respectively after vortex and incubation each time. The homogenate was extracted with phenol-chloroform-iso-amyl alcohol at 12000 rpm for 15 min. The supernatant was extracted at 12000 rpm for 15 min after adding 600 µL ice cold isopropanol to the supernatant. Then the pellet was washed with 200 µL 70% ethanol. The air dried DNA sample was suspended in TE buffer (10 mM tris-HCl and 1 mM EDTA at pH 8.0) and stored at -20°C until further analysis. The isolated DNA (5 µL of preparations) from each genotype was run in 0.8% agarose gel for purity and also scanned in spectrophotometer's (Spectronic Genesys, Thermo Scientific) at 260 nm to take the absorbance in order to take quantification of DNA.

Primer selection and RAPD analysis: The pre-screening was done with 23 primers and three arbitrary decamer primers (Operon technologies, USA) namely, OPA01, OPB06 and OPB07 (Table 2), were selected based on their ability to detect distinct polymorphic amplified products of mungbean. The DNA amplification reactions were carried out in a DNA thermocycler (Biometra-4111255, Biotron). Each 10 µL reaction mixture contained about 50 ng of template DNA, 10X Ampli *Taq* polymerase buffer, 250 µM dNTP mix, 10 µM primer and 0.2 µL *Taq* DNA polymerase. The thermal cycling conditions were, 3 min preheat at 94°C, 40 cycles of 1 min at 94°C, 1 min annealing at 54°C and elongation or extension at 72°C for 2 min. Finally, all amplified fragments were allowed for 7 min at 72°C to complete extension and temperature was brought down to 4°C. The RAPD fragments were separated by performing 1.4% agarose gel electrophoresis and the gel was documented using UV transilluminator in a gel doc (Biometra, Japan). All the PCR reactions were repeated at least twice to check the reproducibility.

Scoring and data analysis: Positions of clearly visible and scorable RAPD bands of all genotypes were thereby given identification numbers according to their position on gel and scored visually with 1 for presence and 0 for absence at particular position for each individual genotype against each primer. The 1000 bp DNA ladder was used as standards for estimating the molecular weights of the bands. All amplifications were repeated at least twice to get reproducible bands for further analyses. A single data matrix was created using the scores obtained from the RAPD analysis and it was used to determine polymorphic loci, Nei (1973) gene diversity, population differentiation (G_{st}), gene flow (N_m), genetic distance and to construct a Unweighted Pair Group Method of Arithmetic means (UPGMA) dendrogram using POPGENE computer program, version 1.31 (Yeh *et al.*, 1999). The pair-wise homogeneity test was also performed using the same program throughout different loci.

The assumption of a two-allele system was the basis for gene frequency estimation for RAPD loci. It was used to calculate the Nei's genetic distance (Nei, 1972, 1978) from the RAPD pattern. The Similarity Index (SI) of any two individuals on the same gel of the RAPD profiles was calculated manually according to:

$$SI = \frac{2N_{xy}}{(N_x + N_y)}$$

where, N_{xy} is the number of RAPD bands shared by individuals x and y, respectively and N_x and N_y are the number of bands in individual x and y, respectively (Lynch, 1990). The SI value ranges from 0 to 1. When SI = 1.0, the two DNA profiles are identical and when SI is 0.0, there are no common bands between the two profiles. The similarity within population (S_i) and similarity between populations (S_{ij}) were calculated as the average of SI across all possible comparisons between individuals within a population and as the average

Table 2: Primers with their sequences used for RAPD analysis and corresponding number of RAPD DNA merkers generated in 10 mungbean genotypes

Primer code	Primer sequences (5´-3´)	GC content (%)	Total bands	Size ranges (bp)	Polymorphic bands	Polymorphic loci (%)
OPA01	CAGGCCCTTC	70	5	300-700	5	100
OPB06	TGCTCTGCCC	70	5	350-900	3	60
OPB07	GGTGACGCAG	70	8	100-920	6	75
Total			18		14	235
Average			6		4.67	78.33

similarity between randomly paired individuals from population i and j, respectively (Lynch, 1991). Gene flow (N_m) was estimated according to:

$$N_m = 0.5\frac{(1 - G_{st})}{G_{st}}$$

where, G_{st} is the proportion of total genetic diversity attributable to subpopulation. It is also known as coefficient of gene differentiation. The G_{st} value was calculated using the following equation:

$$G_{st} = 1 - \frac{H_s}{H_t}$$

where, H_s is the mean average heterozygosity of the total population and H_t is the mean of Hardy-Weinberg expectation of heterozygosity obtained with population average allele frequencies.

RESULTS AND DISCUSSION

The objective of the present study was to evaluate the extent of genetic variability and phylogenetic relationships among the varieties of mungbean which has been collected from AVRDC and BINA (Table 1). The yield of DNA was 4.65-13.35 μg μL^{-1} tissue. In this study, twenty three primers were screened for their ability to produce polymorphic patterns, resulted in discrete profiles with all the DNAs tested. Three primers among the all primers (OPA01, OPB06 and OPB07) resulted reproducible and distinct polymorphic amplified products and were used with all 10 accessions of mungbean for RAPD analysis. The selected 3 primers produced maximum number of high intensity band with minimal smearing, good technical resolution and sufficient variation among the different cultivars. The representative profile of RAPD products are shown in Fig. 1a-c.

A total of 18 amplification products were scored for these three primers (Table 2) in the 10 varieties of mungbean with an average of 6 bands and 4.67 polymorphic bands per primer. Among the three primers, OPB07 generated maximum eight bands and other two primers OPA01 and OPB06 produced five scorable bands with size range from 100-920 bp. For individual accessions, the highest number of bands were produced by OPB07 in the variety MB09 and MB12 and the lowest number of bands was produced by OPB01 in the variety MB15. Besides these, the primer OPA01 amplified maximum number of polymorphic bands (100%) while the primer OPB06 generated the least (60%) polymorphic bands (Table 2). These results indicated that the RAPD analysis can discriminate the studied varieties of mungbean and the RAPD analysis is considered as a highly sensitive method for the screening of nucleotide sequence polymorphisms that randomly distributed throughout the genome (Subudhi and Huang, 1999).

In the present study, our findings were almost same as of polymorphism detected by the arbitrary primers with the previous findings of other RAPD studies of mungbean genotypes, 6 polymorphic bands per primer (Afzal *et al*., 2004) and of chickpea genotypes, such as 5 polymorphic bands per primer in chickpea accessions (Banerjee *et al*., 1999), 5.52 polymorphic bands per primer in Indian *Cicer* species (Kumar *et al*., 2004). The reasons of the considerable number of average scorable and polymorphic bands could be the presence of 60-70% GC in the used primers (Fukuoka *et al*., 1992). Moreover, the considerable number of average scorable and polymorphic bands was increased with the increasing GC content of the primer. This may be the cause of stability o f nitrogenous base complementation, where G and C have strong (three hydrogen bond) bonds instead of weak (two hydrogen bond) bonds with A and T.

The DNA polymorphisms were detected according to the presence and absence of band. Absence of band may be caused by failure of primer to anneal a site in some individuals due to nucleotide, sequences difference or by insertions or deletions between primer sites (Clark and Lanigan, 1993). Frequencies of maximum number of polymorphic loci were found to be high with the exception of OPB07, OPB06 and OPA01 (Fig. 2). The estimated average value of Nei (1973) gene diversity and Shannon's information index for entire genotypes of mungbean were 0.31±0.19 and 0.45±0.27, respectively. There was a moderate level of genetic variation among the studied genotypes of mungbean from the proportion of polymorphic loci point of view (Fig. 2). Lakhanpaul *et al*. (2000) reported low to moderate level of polymorphisms using 21 RAPD primers for 32 Indian mungbean cultivars.

The resulted amplification products were used as input data for computation of inter-genotype similarity indices (%) and pair wise genetic distance (Table 3). The lowest genetic distance was 0.1178 between the accessions MB09 and MB10, MB10 and MB22, MB21 and MB23 and MB23 and MB32. In contrast, the highest inter-genotype similarity indices (93.73%) were between MB09 and MB10 only. Hence, the genetic similarities indices can be used as basis for the selection of the parents for breeding and mapping populations. The highest genetic distance was 1.0986 between the accessions MB11 and MB12. As a result, the lowest inter-genotype similarity indices (39.05%) were found in these accessions. Moreover, the highest level of inter genotype similarity index (average 77.35%) was generated

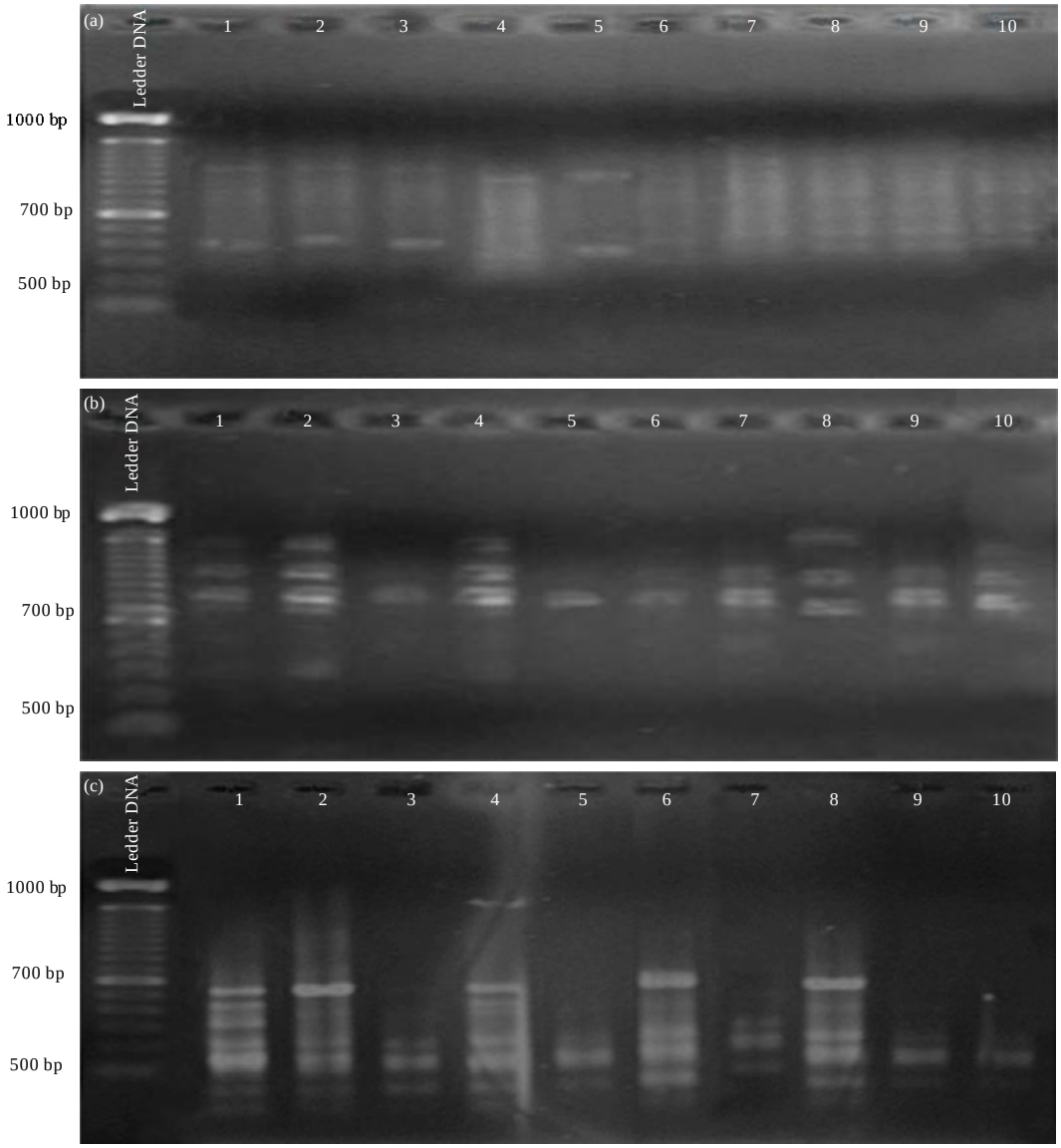

Fig. 1(a-c): RAPD profiles of 10 mungbean genotypes, (a) OPA01, (b) OPB06 and (c) OPB07, Lane M: Molecular marker (1000 bp DNA ladder), Lanes 1-10: Different genotypes of mungbean, 1: MB09, 2: MB10, 3: MB11, 4: MB12, 5: MB13, 6: MB15, 7: MB21, 8: MB22, 9: MB23 and 10: MB32

Table 3: Pair wise inter-genotype similarity indices (%) (above diagonal) and Pair wise genetic distance values (below diagonal) in 10 genotypes of mungbean

Genotypes	MB09	MB10	MB11	MB12	MB13	MB15	MB21	MB22	MB23	MB32
MB09	****	**93.73**	64.44	58.19	40.11	41.88	75.56	82.61	75.56	68.69
MB10	**0.1178**	****	74.6	64.10	41.27	52.38	66.67	88.89	74.08	69.63
MB11	0.4055	0.4055	****	**39.05**	66.67	55.56	66.67	63.49	77.78	64.13
MB12	0.5878	0.4055	**1.0986**	****	65.71	49.82	65.19	86.33	71.85	76.10
MB13	0.8109	0.8109	0.2513	0.5878	****	55.56	55.56	52.38	66.67	64.13
MB15	0.4055	0.4055	0.4055	0.4055	0.2513	****	42.37	52.38	44.45	48.89
MB21	0.4055	0.5878	0.4055	0.8109	0.5878	0.8109	****	77.78	88.89	80.95
MB22	0.2513	**0.1178**	0.5878	0.2513	0.8109	0.4055	0.4055	****	85.19	84.87
MB23	0.4055	0.4055	0.2513	0.5878	0.4055	0.5878	**0.1178**	0.2513	****	90.47
MB32	0.4055	0.4055	0.4055	0.4055	0.4055	0.4055	0.2513	0.2513	**0.1178**	****

Data is mean value of three primers-generated values. The genotype names are listed in Table 1

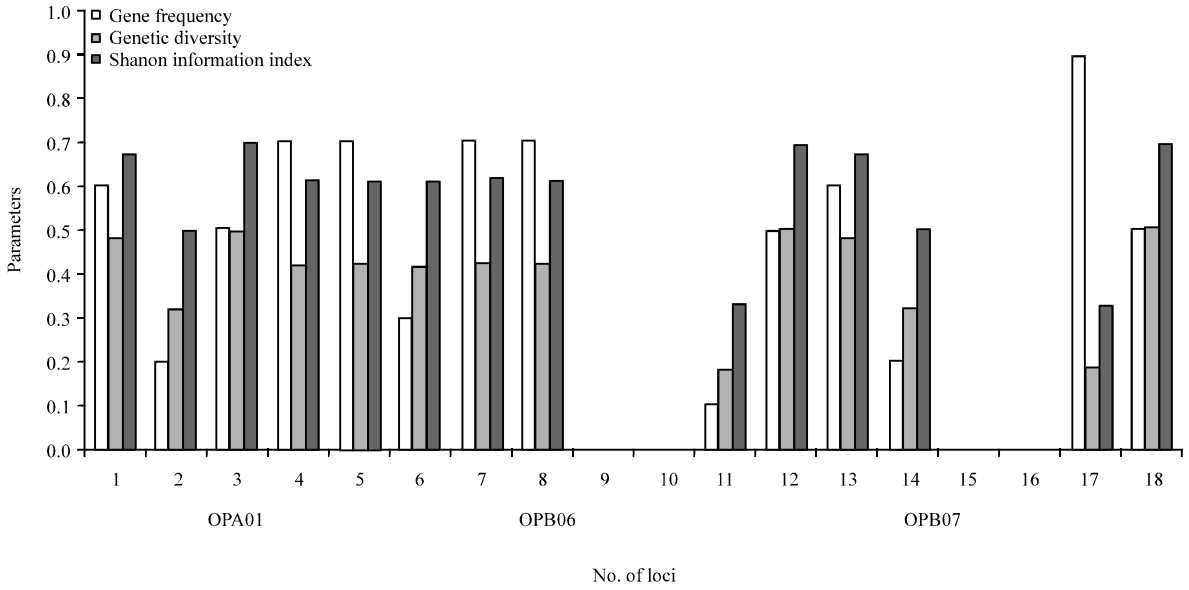

Fig. 2: Estimation of gene frequency, gene diversity and Shanon information index for RAPD primers OPA01 (5: 1-5), OPB06 (5: 6-10) and OPB07 (8: 11-18) of observed mungbean genotypes

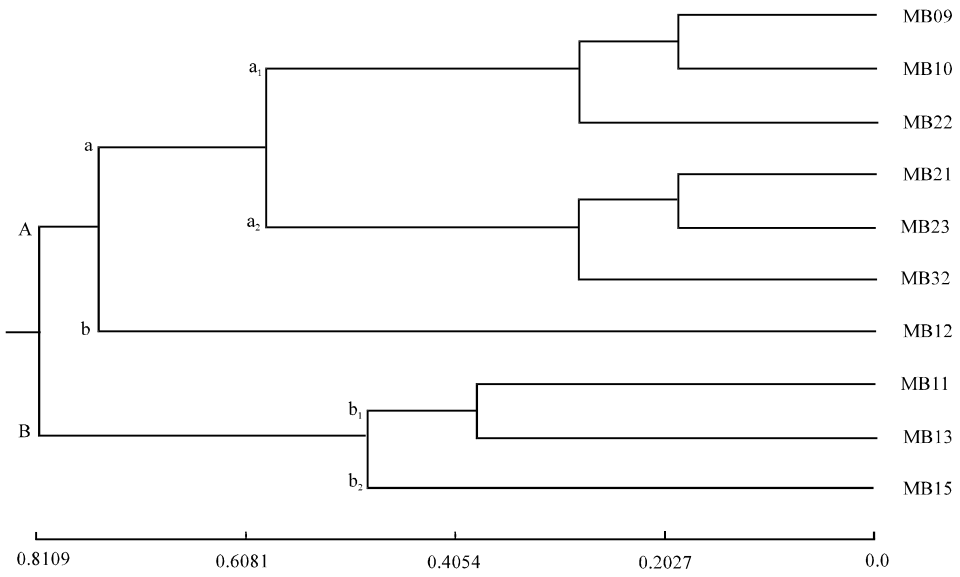

Fig. 3: Dendrogram derived from UPGMA cluster analysis having genetic relationship among 10 Bangladeshi mungbean germplasms based on RAPD analysis

by the primer OPB06. But the lowest inter-genotype similarity index value (average 45.71%) was produced by primer OPA01. Considering the similarity indices or genetic distance values (Table 3), the results indicated that some genotypes of mungbean are genetically different from each other and some tend to be similar.

Nei's (1973) analysis of gene diversity in subdivided populations presented the gene flow (N_m^+) value of 0.0 and the proportion of total genetic diversity (G_{st}) was 1.00. Hardy-Weinberg expectation of average heterozygosity in sub-population (H_t) was 0.3089, whereas the heterozygosity (H_s) was 0.00 (30). The UPGMA dendrogram (Fig. 3) was constructed based on Nei's genetic distance of three primers scorable fragments varied from 0.1824-0.8109 in order to show the relationship between species and varieties and clearly delineated all

10 mungbean genotypes. The combined dandogram of 10 genotypes of mungbean were divided into 2 main clusters namely cluster A and B (Fig. 3). The cluster A had 2 sub-clusters (a and b), the sub-cluster 'a' contained genotypes MB09, MB10, MB22, MB21, MB23 and MB32 while the sub-cluster 'b' contained genotypes MB12 along with MB09 and MB32 distinct genotype in the cluster A. Sub-cluster 'a' comprised of two sub-sub-clusters (a_1 and a_2) with genotypes MB09, MB10 and MB22 in a_1 sub-sub-cluster while genotypes MB21, MB23 and MB32 in a_2 sub-su b-cluster. Sub-sub-cluster a_1 comprised another to sub-sub-sub-cluster with showing strong relations between MB09 and MB10 in first sub-sub-sub-cluster along with MB22 as a distinct genotype in the second sub-sub-sub-cluster of a_1. Similarly, sub-sub-cluster a_2 comprised another two sub-sub-sub-clusters with showing strong relationships between MB21 and MB23 in first sub-sub-sub-cluster along with MB32 as a distinct genotype in the second sub-sub-sub-cluster of a_1. Cluster B comprised of MB11 and MB13 along with MB15 as a distinct genotypes of B cluster. On the basis of UPGMA dandogram, genotypes MB11, MB12 and MB13 were found to be quite distinct and they can be used for improvement of mungbean cultivars in breeding purposes. 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.

In the few earlier studies, the lack of correlation (Bisht *et al*., 1998; Lavanya *et al*., 2008; Manivannan *et al*., 1998) or partial correlation (Raturi *et al*., 2012) between geographical origin and genetic variability in mungbean has been reported. However, the RAPD analysis technique may get the important considerations for the speed, efficiency, accuracy and reliability for the development of mungbean germplasms.

CONCLUSION

The present study would be effective for the identification of the parents genotypes based on their characteristics of high yielding in order to develop the high yielding new variety using hybridization, gene transfer technology or any other relevant molecular techniques. Therefore it can be concluded that for further research program, especially for hybridization, the aimed genotype could be selected from different clusters that might be useful for the evolution of desired genotypes.

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