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Genetic Diversity Analysis of BARI Masur Varieties of Lentil (*Lens culinaris* Medik.) based on RAPD Markers

Subroto K. Das*, Mst. Mim, Sonia Khan Sony, Shawon Mitra, Shahela Akter[1] and R. H. Sarker[2]

Department of Botany, University of Barishal, Barishal-8254, Bangladesh

Key words: Lentil, RAPD, Genetic diversity, Phylogenetic relationship

Abstract

Genetic diversity analysis of seven BARI masur (BM) lentil varieties from Bangladesh was performed using polymerase chain reaction (PCR) with the random amplification of polymorphic DNA (RAPD) technique. Seventy-six unique bands were obtained using 16 of the 20 randomly selected decamer oligonucleotide primers, with an average of 4.75 bands per primer. Ten primers were found to be 100% polymorphic based on the observed banding patterns. Significant genetic variation is evident, as evidenced by pairwise genetic distances ranging from 0.1563 to 0.6931. Seven lentil varieties have been divided into three main clusters via a dendrogram based on Nei's genetic distance using the Unweighted Pair Group Method of Arithmetic Means (UPGMA), with a similarity coefficient of 0.5198. Cluster 1 consisted of BM-1, BM-2, BM-3 and BM-5, cluster 2 consisted of BM-4 and BM-7 whereas only BM-6 was found in cluster 3. Cluster 1 was further grouped into three sub-clusters and the varieties BM-1 and BM-2 were placed in the same sub-cluster, whereas the varieties BM-3 and BM-5 were placed in separate sub-clusters. The varieties BM-1 and BM-2 showed relatively less divergence among the varieties of the cluster 1. Conversely, BM-6 variety was presented in a separate cluster that indicates this variety is more divergent than the other varieties.

Introduction

Lentil (*Lens culinaris* Medik.) is an annual self-pollinating diploid (2n=2x=14) crop species. As old as emmer, peas and barley, lentils are among the first domesticated plant species. This crop's genome is ~4063 Mbp in size. Lentils are considered to have originated specifically in Egypt and later, they moved to Latin America.

As one of the first crops cultivated in the Near East, lentils have been a part of human diets since the Neolithic era. They remain one of the most significant cool-season annual grain legumes or pulse crops globally. Bangladesh ranks sixth position in the world's

*****Author for correspondence: <mrsubroto@yahoo.com>. [1]Military Collegiate School Khulna (MCSK), Bangladesh. [2]Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh.

total lentil production (FAO 2023). In Bangladesh it is cultivated in 2.493 hectares of land producing 269 thousand metric tons/annum and having an average yield of 1.080 tons per hectare. Bangladesh ranks first in terms of customer preference among pulse crops, despite being the second-most productive crop in terms of area (BBS 2023). In Bangladesh, lentils are one of the most significant pulses crop due to their application in farming systems, food, and feed (Ganesan and Xu 2017). Lentil is a highly protein-enriched pulse crop, and about twice as much protein is found in it as in cereals (Alexander et al. 2024).

Globally, consumers choose it over other pulses varieties. As a source of protein for human and animal nutrition, this significant grain legume has gained economic significance on a global scale. The Indian subcontinent steadily increased demand for this crop due to its high nutritional value, excellent cooking quality, and simple digestibility. Globally, the pace of increase in lentil consumption is more than twice that of the growth in the human population. We predict a twofold increase in lentil consumption worldwide by 2030.

In Bangladesh, it is extensively cultivated in Jashore (28,003 acres), Faridpur (42,704 acres) and Rajshahi (33,145 acres) (BBS 2023). In Bangladesh, the production of local native lentil varieties is relatively low and varies significantly between fields and localities (Miah et al. 2021). The Bangladesh Agricultural Research Institute (BARI) has developed several well-known lentil varieties which grow in the fields owned by the farmers. However, researchers and policymakers are unaware of this crop's adoption status and economic performance, due to inadequately developed molecular techniques for genetic and genomic analysis of lentils. Therefore, there is an urgent need to increase the availability of high-quality molecular genetic markers for use in breeding programs that employ marker-assisted selection (MAS). As well as enhancing biotechnology capabilities through the development of information resources in important legume species, such as lentils.

It has been demonstrated that numerous molecular markers, for instance, RAPD, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSRs), are effective tools for the characterization of agricultural crops based on genetic diversity. Among the mentioned molecular techniques, RAPD is highly reliable, quicker, and user-friendly for identifying and tracking pedigree breeding records of inbred varieties (Baruah et al. 2017, Cheula et al. 2020, Makmur et al. 2020, Kamble et al. 2023). It also helps determine the genetic relationships between genotypes and their wild forms (Cattan-Toupance et al. 1998). With the aid of this method, genomic variation can always be examined without requiring prior knowledge of DNA sequences. It is beneficial in revealing the differences between individuals when other methods are unable to do so for species with low genetic variability.

Furthermore, several scientists have categorized and asses the relatedness and diversity of agricultural genotypes employing RAPD (Porreca et al. 2001, Rabbani et al.

2008). Moreover, in economically significant crops, RAPD fingerprinting provides a precise estimation of varietal distinctiveness and irrelativeness (Mitra et al. 2017). Restriction sites are no longer necessary with this updated method of plant varietal identification, which detects polymorphisms using PCR technology (Welsh and McClelland 1990). By utilizing highly specific and sensitive oligonucleotide primers, this strategy maps traits and fingerprints individuals for the advancement of crops (Quershi et al. 2025, Jha et al. 2024, Deshahalli et al. 2024, Dutta et al. 2024, Gore et al. 2022).

Plant germplasm collection and preservation have become even more crucial due to the destruction of habitats triggered by modern agriculture and genetic erosion. Landraces with an immense genetic diversity are distributed in distant villages in several countries, including Bangladesh. Nevertheless, due to introduction of high-yielding cultivars the number of landraces began to decrease. Most of the ancient landraces are no longer in the hands of farmers but rather exist solely in certain gene banks. Rationalizing genetic resource conservation and utilization is quintessential in order to maintain genetic diversity employing advanced molecular techniques, which is imperative for molecular plant breeding.

Considering the significance of lentils in Bangladesh's agriculture and the limitations of traditional breeding methods for increasing yield and quality, it is necessary to find desired characteristic genes within the varieties to enhance them. Therefore, the present investigation aims to characterize lentil varieties in Bangladesh, focusing on genetic diversity assessment employing RAPD techniques for efficient crop improvement.

Materials and Methods

Seeds of seven cultivated microspermae varieties of lentil known as, BARI Masur-1 (BM-1), BARI Masur-2 (BM-2), BARI Masur-3 (BM-3), BARI Masur-4 (BM-4), BARI Masur-5 (BM-5), BARI Masur-6 (BM-6), and BARI Masur-7 (BM-7) were used in this experiment. These cultivated lentil varieties were released by Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh. Additionally, 20 different types of decamer primer were used, including A03, A08, A09, A15, B06, B14, C01, C02, D01, D02, OPA1, OPA2, OPA3, OPA4, OPA5, OPA6, OPA7, OPA8, OPA9, and OPA10. The plants were raised and maintained in pots at the Department of Botany, University of Barishal.

30 days old pot-grown seedlings fresh young leaves were collected in order to extract genomic DNA (gDNA) using CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle 1987). To get rid of any additional foreign DNA sources and microbe spores, the leaves were cleaned in distilled water and ethanol and then dried on fresh tissue paper. Pulverized 200 mg of leaf tissue, 1.6 ml of extraction buffer was added, containing 3% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, and 0.2% mercaptoethanol. The homogenous paste of leaves was then moved to a 2.0 ml Eppendorf tube and incubated for 30 min at 60°C in a water bath. The samples were centrifuged at 13,000 rpm for 10 min at 4°C (thermo eppendorf, GK bioscience, India) then a 24 : 1 volume of chloroform : isoamyl alcohol was added to the supernatants and

centrifuged again. The DNA was precipitated overnight (the supernatant with 2/3 vol. chilled isopropanol), then centrifuged for 10 min at 13,000 rpm, and the DNA pellet was collected. The pellet was cleaned with 70% ice-cold ethanol, air dried, and then dissolved in 50µl of TE buffer, treated with RNase A for 30 min, and stored at -20°C.

Sixteen decamer primers with 60–70% G+C content was utilized for RAPD analysis. The PCR reaction mixture (25 µl), consisting of 10 × Taq DNA buffer (2.5 µl), 100 µM dNTPs, 1 µM primer, 1U Taq polymerase (Thermo Scientific), and 50 ng gDNA and maintained at 4°C during preparation. The PCR amplification mixture was put in a thermal cycler (Applied Biosystems) with the following settings: 94°C (5 min), 35 cycles of 94°C (45 sec), 32 or 34°C (30 sec), 72°C (3 min), and one cycle of 72°C (7 min). Following the cycling programme, the reactions were maintained at 4°C. To ensure the integrity of the results, a negative control (water instead of template) was added to each thermal cycle, effectively preventing any external contamination of the amplification products.

The PCR amplified products were separated electrophoretically on 1% agarose gel with 1 ×TAE buffer and ethidium bromide, conducted at 90 volts and 250 mA for 45 min. Alongside the amplified product, a single molecular weight marker-1 Kb DNA ladder (Gene RulerTM), was electrophoresed. To ensure the reproducibility of the results, PCR reactions were performed on the first DNA extract for each variety at least twice, and the results were verified using a separate DNA extract from the same variety. For each of the seven varieties and 20 primers, the amplified products were evaluated based on the presence or absence of bands, for each of the seven varieties and 20 primers, faint bands were ignored.

A UV Trans illuminator (Cleaver Scientific Ltd.) was used to photograph the gel. The binary matrix was generated by selecting high-intensity and well-separated bands, indicating the presence (1) or absence (0) of specific RAPD bands. According to Jaccard's coefficient pairwise comparisons were calculated (Jaccard 1908). The unweighted pair group method analysis (UPGMA) was used to create a dendrogram and calculate the correlation between the clusters' cophenetic matrix and similarity matrix. Each RAPD primer's polymorphic information content (PIC) value was determined using the following formula: PIC= 1-Σ(Pij)2, where Pij = ith pattern's frequency revealed by the jth primer added to all other patterns yielded by the primers (Botstein et al. 1980). The assessment of genetic variation can help to prevent duplications and streamlining of genotypes, aiding in the advancement of our lentil varieties.

Results and Discussion

The objective of the current study was to assess the genetic variations across the seven lentil varieties using a total of 20 random decamer primers by reproducible DNA amplification employing RAPD analysis. However, four of these primers (A09, B14, D02, and OPA4) were unable to generate a dark, distinguishable band, whereas the rest of the sixteen primers yielded reproducible bands using the same PCR technique. By

repeating the identical PCR reaction twice each primer's reproducibility was verified. Seven distinct lentil varieties yielded 76 repeatable and scoreable amplification (1 to 11) results using 16 primers with an average of 4.75 bands per primer. 61 (80.3%) of the 76 bands were discovered to be polymorphic (with 1 to 11 fragments per primer) for one or more varieties. The maximum percentage of polymorphic bands was found using OPA01, OPA02, OPA03, OPA06, 0PA07, OPA09, A15, B06, C01 and D01 primers, whereas the primer A03 yielded a minimum number (25%) of polymorphic bands. The primer OPA10 shows no polymorphic bands among the varieties (Table 1).

Table 1. Analysis of reproducible loci with different RAPD primers within the lentil varieties.

Primers	Sequences (5′–3′)	Total no. of RAPD Loci	No. of polymorphic RAPD Loci	% of polymorphism
OPA01	TGC CGA GCT C	5	5	100
OPA02	TGC CGA GCT G	3	3	100
OPA03	AGT CAG CCA C	5	5	100
OPA05	AGG GGT CTT G	8	5	62.5
OPA06	GGT CCC TGA C	4	4	100
OPA07	GAA ACG GGT G	11	11	100
OPA08	GTG ACG TAG G	7	6	85.71
OPA09	GTG ATC GCA G	6	6	100
OPA10	GTG ATC GCA G	3	0	0
A03	AGT CAG CCA C	4	1	25
A08	GTG ACG TAG G	5	2	40
A15	TTC CGA ACC C	1	1	100
B06	TGC TCT GCC C	3	3	100
C01	TTC GAG CCA G	3	3	100
C02	GTG AGG CGT C	3	1	66.67
D01	ACC GCG AAG G	5	5	100

Previous works on lentils showed a considerable polymorphism using molecular marker-based characterization. Numerous scientists (Tayyaba 2003, Rana et al. 2007, Gupta et al. 2012, Hoque and Hasan 2012) were done molecular experiments on different varieties of lentils to evaluate the genetic divergence and found 50, 42.3 and 60.37% polymorphism, respectively. In this investigation it is observed that, 80.3% polymorphic bands among the seven varieties which were released from the BARI. This finding indicates a diverge genetic variation within the analyzed lentil varieties.

A wide range of polymorphisms have been identified in many crops employing RAPD markers, including 72.2% in maize (Carvalho et al. 2004), 49.12% in wheat (Nimbal et al. 2009), 61% in canola (Moghaieb et al. 2014), 94.168% in tomato (Tabassum et al. 2013), 100% in mungbean (Sony et al. 2013), 84.44% in cabbage (Saxena et al. 2011). The study also demonstrated the importance and significance of the banding profiling of

OPA01, OPA02, OPA03, OPA05, OPA06, OPA07, OPA08, OPA 09, OPA10, A03, A08, A15, B06, C01, C02 and D01 primers in evaluating and identifying polymorphism dependencies in variety-specific unique bands. These primers resulted in ten unique bands within the BM-3, BM-4, BM-5, and BM-7 varieties and the number of bands were 1, 2, 2, and 5, respectively (Fig. 1).

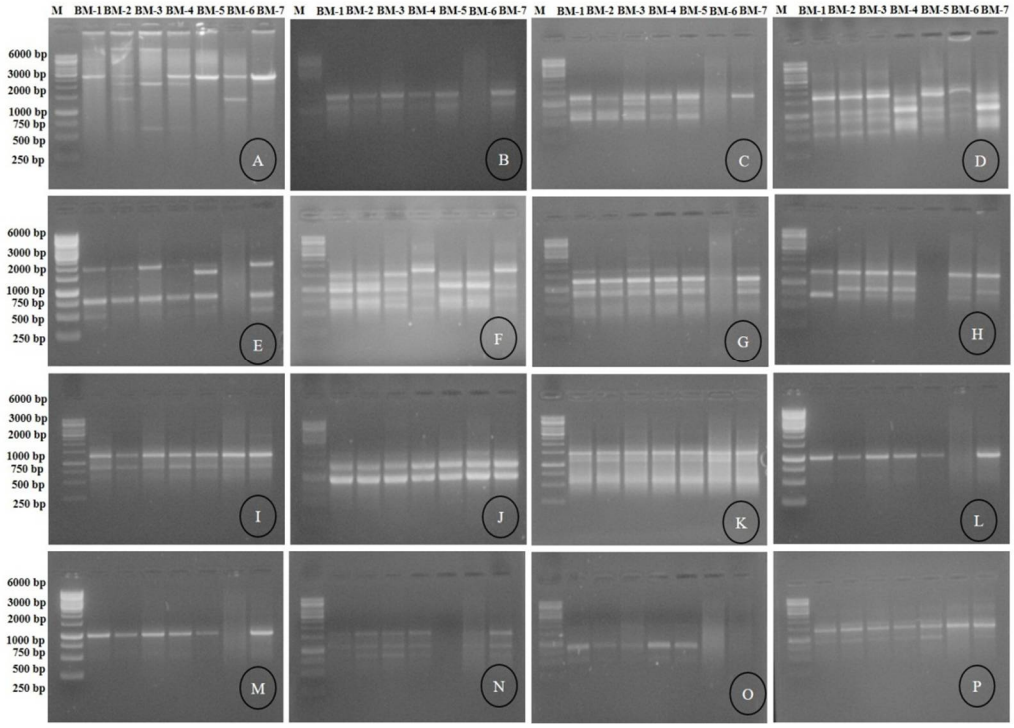

Fig. 1. RAPD profile of seven lentil varieties obtained with the primers OPA01 (A), OPA02 (B), OPA03 (C), OPA05 (D), OPA06 (E), OPA07 (F), OPA08 (G), OPA 09 (H), OPA10 (I), A03 (J), A08 (K), A15 (L), B06 (M), C01 (N), C02 (O), D01 (P). Lane- M 1Kb DNA Marker and BM-1, BM-2, BM-3, BM-4, BM-5, BM-6, BM-7 indicates BARI Masur 1, BARI Masur 2, BARI Masur 3, BARI Masur 4, BARI Masur 5, BARI Masur 6, and BARI Masur 7 variety of lentil respectively.

A genetic distance matrix was constructed using RAPD profiling to ascertain the level of divergence within and between seven lentil varieties. The Nei's distance matrix was generated to estimate the divergence of the lentil varieties and revealing a range of 0.1563 to 0.6931 (Table 2).

The minimum index value was observed between BM-1 and BM-2 varieties, whereas the maximum index value was between BM-4 and BM-6. These results point out that the variety BM-1 and BM-2 are closely related. On contrary, BM-4 and BM-6 are distantly related. The minimum and maximum index values range indicates a considerable variability persisting within and among the seven lentil varieties.

Table 2. Summary of genetic distances matrix in seven lentil varieties (Nei's 1972).

Sl. No.	1	2	3	4	5	6	7
1	0						
2	0.1563	0					
3	0.2036	0.2199	0				
4	0.4187	0.3604	0.4187	0			
5	0.2199	0.3054	0.3234	0.4806	0		
6	0.4187	0.3604	0.5465	0.6931	0.5241	0	
7	0.4806	0.4595	0.4806	0.3234	0.5465	0.6672	0

A dendrogram depicting the varieties in a clustering manner was developed using genetic distance based on RAPD data. The dendrogram was constructed based on Nei's genetic distance matrix (Nei 1972) employing UPGMA, which categorized seven lentil varieties into three main clusters with a similarity coefficient of 0.5198. BM-1, BM-2, BM-3, and BM-5 made up Cluster 1, BM-4 and BM-7 made up Cluster 2, whereas only BM-6 located in the cluster 3. The cluster 1 was further grouped into 3 sub-cluster, and the varieties BM-1 and BM-2 placed in same sub-cluster, whereas the varieties BM-3 and BM-5 were placed in separate sub-cluster. The BM-1 and BM-2 varieties in cluster 1 exhibit less divergence due to their origins from closely related ancestors. However, the BM-6 variety presented in a separate cluster that indicates this variety is more divergent than other varieties (Fig. 2).

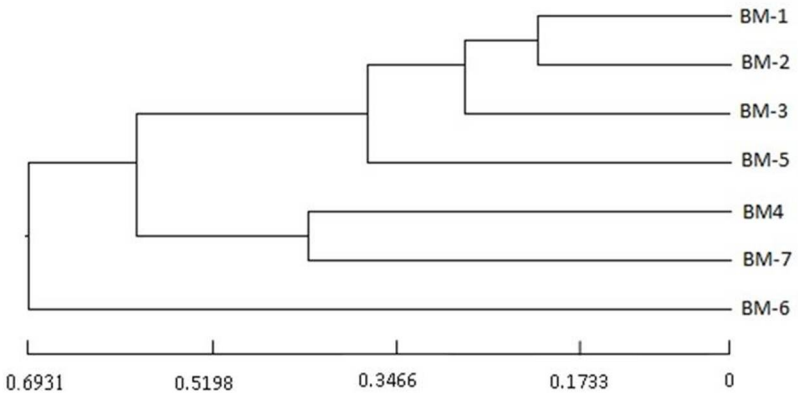

Fig. 2. Tree diagram for 7 lentil varieties of Bangladesh based on unweighted pair of arithmetic mean (UPGMA).

The present investigation demonstrated that RAPD markers, along with appropriate statistical methods, were successfully applied to study the phylogenetic relationships among the varieties. Furthermore, the distinct bands found for seven lentil varieties in this study demonstrate the effectiveness of RAPD markers in genetic diversity analysis and fingerprinting. This study is nearly identical to previous research (Boonerjee et al. 2013, Islam et al. 2017, Sweety et al. 2017, Rahman et al. 2017) where RAPD analysis was performed to examine the evolutionary relationships among the genotypes of the crop.

The recent effort has made it feasible to determine the genetic variation and relatedness among the seven lentil varieties, released from the BARI. Genetic variability is a crucial factor in successful crop improvement programs (Ravi et al. 2003). The assessment of Genetic diversity employing RAPD markers aided with the genotype characterization process.

On the basis of RAPD data cluster analysis revealed little genetic difference amongst multigrain varieties despite their similar morphological form. Furthermore, the current investigation demonstrated that lentil varieties in the first cluster share a significant level of genetic similarity, maybe because of deriving from closely related ancestors. Moreover, the present findings have made it possible to develop a molecular genetic map, which could be utilized by marker-assisted selection tools for lentil genetic improvement.

In this study, a unique approach was performed by utilizing sixteen RAPD primers in employing PCR to assess the genetic variation of between and within seven lentil varieties (BM-1, BM-2, BM-3, BM-4, BM-5, BM-6 and BM-7). RAPD markers have shown to be an effective tool in molecular genetic analysis of lentil cultivars, aiding in plant breeding programs to assess genetic diversity and develop improved varieties able to withstand biotic and abiotic stresses. This study assesses genetic variation and relatedness of lentil varieties in Bangladesh using a limited number of varieties and primers. Moreover, it is recommending further research with a broad spectrum of economically important crops.

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