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Assessment of Genetic Variability among Six Bangladeshi Foxtail Millet (*Setaria italica* L.) Varieties using RAPD Markers

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Key words: Foxtail millet, Genetic diversity, RAPD markers, Plant breeding, Food security

Abstract

Foxtail millet (*Setaria italica* L.) is an ancient cereal crop known for its nutritional and medicinal benefits and for being highly adaptable to arid and semi-arid environments. Six varieties of foxtail millet were collected from Bangladesh Agricultural Research Institute (BARI) and different regions of Bangladesh for the assessment of their genetic variability employing Random Amplified Polymorphic DNA (RAPD) markers. This study generated a distinct genetic variability for the varieties, generating 299 bands with the fifteen RAPD primers of size range of 210 bp to 3000 bp with an average polymorphism rate of 97.06%. Among the six varieties, V_2 (BARI Kaon-2) was found to be the most polymorphic with 98.75%, and V_1 (BARI Kaon-1) was the least polymorphic with 91.66%. The cluster and dendrogram analyses further classified the varieties into distinct groups based on their genetic relationships. The genetic distance across the varieties ranged from 0.1865 to 0.9146. The closest relationship was observed between V_5 (Local Variety-Manikganj) and V_6 (Local Variety-Bhola), whereas V_3 (BARI Kaon-3) and V_4 (BARI Kaon-4) were found to be the most genetically distinct. The findings of the present study emphasize the effectiveness of RAPD markers for identifying genetic variation and provide significant information for future foxtail millet breeding programs that supports sustainable agriculture systems.

Introduction

The global agricultural sector faces diverse and significant challenges, especially due to the impact of climate change, population growth, and the insufficiency of sustainable food production systems. It is crucial to ensure food security and environmental sustainability all over the world, especially in regions with water scarcity and poor soil quality. Although, the developments in agricultural practices, including the Green Revolution led to higher crop yields, but also to an over-reliance on a few staple cereals such as rice and wheat. Consequently, there has been poor nutrient intake due to lack of

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dietary diversity. Thus, there has been an increasing demand for alternative crops with high nutritional value resource-efficient, and stress tolerance (Pinstrup-Andersen 2009, Godfray et al. 2010).

These challenges can be mitigated by numerous types of small-seeded cereal crops, including millets, that are stress-resilient, adaptable to marginal soils and grow under conditions of minimal water input. These cereals are loaded with high level of nutrition (protein, vitamins, and minerals), and also providing plenty of fiber. Among millets, foxtail millet (*Setaria italica* L.) is an important millet as it is drought tolerant and can enhance the food security in the areas affected by climate change (Jain et al. 2016, Muthamilarasan and Prasad 2016).

Foxtail millet is one of the oldest cultivated cereal crops and has been cultivated in many parts of Asia and other semi-arid areas. This crop has been categorized as a valuable small millet because of its ability to survive in adverse environmental conditions, short life cycle, and minimal inputs of resources (Skot and Stamoulis 2006). In Bangladesh, it is known as Kaon locally. Recently, foxtail millet has gained global attention for its nutritional richness (carbohydrates, proteins, dietary fiber, vitamins, and essential minerals) and its potential contribution to food and nutritional security under changing climatic conditions (Godfray et al. 2010). They possess low glycemic value and hence is useful for diabetic patients (Chandrasekara and Shahidi 2012). However very limited research has been conducted about its genetic diversity and agronomic characteristics, especially in developing countries such as Bangladesh, where the use of foxtail millet has remained neglected (Lata et al. 2013, Saleh et al. 2013). Exploring the genetic variation of foxtail millet through molecular approaches is quintessential for increasing its crop production and nutritional value.

Genetic diversity is an important aspect for crop improvement, conservation, and adaptation to diverse environmental conditions. The presence of genetically diverse germplasm offers potential opportunities to generate improved varieties with desirable traits, for instance, higher productivity, stress tolerance, and disease resistance. Thus, the assessment of genetic variation of germplasm is essential for successful breeding programs (Das et al. 2025). Conventional morphological traits-based characterization is generally influenced by environmental conditions and thus does not represent an accurate genetic relationship between genotypes (Govindaraj et al. 2015). Therefore, molecular marker-based techniques have proven to be a reliable tool for the estimation of genetic variability at DNA level (Sony et al. 2013, Mollick et al. 2025). Among the different types of molecular marker, RAPD-based markers are utilized extensively in numerous crop species, as they are simple, cost-effective, rapid, and can detect polymorphisms without sequence data (Sony et al. 2013, Mitra et al. 2017, Dutta et al. 2025). Some local foxtail millet landraces are grown traditionally in Bangladesh but information regarding their molecular variability is insufficient. The gradual replacement of indigenous varieties by modern crops may lead to the loss of valuable genetic resources.

By leveraging molecular breeding techniques such as RAPD markers, this research aims to accelerate the identification of nutritionally improved high-yielding, stress-tolerant foxtail millet varieties. This work could provide valuable insights for germplasm conservation and future breeding programs focused on developing improved foxtail millet cultivars, which may play a pivotal role in addressing future food security challenges.

Materials and Methods

The present study involved six different varieties of foxtail millet, which were collected from various regions of Bangladesh. Among these, four improved or high-yielding varieties released by the BARI, were included: V_1 (BARI Kaon-1), V_2 (BARI Kaon-2), V_3 (BARI Kaon-3), and V_4 (BARI Kaon-4). The remaining two accessions V_5 (Local Variety-Manikganj) and V_6 (Local Variety-Bhola) were local varieties collected from Ghior, Manikganj and Char Fasson, Bhola, respectively and were named according to their source of collection. All seeds were sown and maintained under controlled conditions in the net house of the Department of Botany at the University of Barishal, Bangladesh. The collected germplasm was utilized for molecular characterization and assessment of genetic diversity using RAPD markers.

Genomic DNA was extracted from young, fresh leaves by using modified CTAB method (Doyle and Doyle 1987). For each variety, leaf samples were collected from ten independent plants and DNA was extracted separately from each plant. RAPD amplification for each primer was performed thrice to confirm the reproducibility of the amplification profiles. Only clear, distinct, and reproducible bands were scored for genetic diversity analysis. The concentration of DNA and purity were determined using a spectrophotometer (T60 U Spectrophotometer, India). DNA quality was further assessed by electrophoresis on a 0.8% agarose gel stained with ethidium bromide, and only high-quality DNA showing intact bands with A_{260}/A_{280} ratios between 1.8 and 2.0 was used for RAPD analysis. For the molecular analysis, 15 decamer RAPD primers were applied (Table 1). The PCR reaction mixture consisted of 25 µl total volume containing 2.0 µl of template DNA (25 ng), 18.8 µl of deionized distilled water, 2.5 µl of 10X Taq buffer (with 1.5 mM MgCl2), 1.0 µl of primer, 0.5 µl of 10 mM dNTP mix, and 0.5 µl of Taq DNA polymerase. PCR amplification was performed in an oil-free Mini Amp thermal cycler (Applied Biosystems, Singapore) for 35 cycles, following these cycling conditions: 94°C for 45 sec, 32 or 34°C for 30 sec, and 72°C for 3 min, followed by one cycle of 72°C for 7 min. Upon completion of the cycling program, the reactions were held at 4°C. The amplified products were then separated on a 1% agarose gel using electrophoresis, made with 100 ml of 1× TAE buffer and 1 gram of agarose powder containing ethidium bromide at a voltage of 100 volts for 30 mins.

The DNA ladder (1 kb plus) was run simultaneously with the sample. The separated DNA bands were observed under a UV Transilluminator (Cleaver Scientific Ltd.). Visualization of the separated DNA bands were done under a UV transilluminator

(Cleaver Scientific Ltd.), and the images were recorded using a Gel Documentation System (FAS-Digi, Germany). Distinct RAPD bands were assigned for identification numbers and scored for presence (1) or absence (0) using "NTSYSpc V.2.10" software (Sharifi et al. 2018). Genetic similarity within the varieties was determined employing the genetic similarity coefficient of Nei, and the values of genetic distances were calculated according to Nei (1972). The obtained data was utilized to generate a UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram depicting the genetic relationship among the foxtail millet varieties.

Table 1. The features of fifteen different RAPD primers employed in this study.

Serial number	Primer	Annealing temperature (°C)
1	OPA02 (5'- TGC CGA GCT G 3')	34
2	OPA03 (5'- ATG CAG CCA C 3')	32
3	OPA04 (5'- AAT CGG GCT G 3')	32
4	OPA06 (5'- GGT CCC TGA C 3')	34
5	OPA07 (5'- GAA ACG GGT G 3')	32
6	OPA08 (5'- GTG ACG TAG G 3')	32
7	OPA09 (5'- GGG TAA CGC C 3')	32
8	OPA10 (5'- GTG ATC GCG T 3')	32
9	OPB01 (5'- GTT TCG CTC C 3')	32
10	OPB04 (5'- GGA CTG GAG T 3')	32
11	OPB05 (5'- TGC GCC CTT C 3')	34
12	OPB06 (5'- TGC TCT GCC C 3')	34
13	OPB07 (5'- GGT GAC GCA G 3')	34
14	OPB08 (5'- GTC CAC ACG G 3')	34
15	OPB10 (5'- CTG CTG GGA C 3')	34

Results and Discussion

Foxtail millet is an important underutilized cereal crop; hence, identification of germplasm containing genetic diversity is essential for crop improvement and conservation. Research on genetic variability provide valuable information on trait inheritance, adaptation, and breeding. Molecular markers are applied to differentiate between various varieties and evolutionary relationship among themselves. DNA markers represent an innovative approach that utilizes DNA polymorphism to support biological research.

Scientists employed various techniques, including morphological characterization, cross-compatibility test, karyotyping, chromosome banding, in-situ hybridization, and molecular markers, to explore the phylogenetic links between different millet species (Banerjee et al. 2020). In this study, molecular analysis was conducted among six Bangladeshi foxtail millet varieties using 20 random primers. Out of these, 15 primers that produced clear amplification products (Fig. 1) were selected to assess the genetic relationships among the foxtail millet accessions. These fifteen primers generated 299 distinct amplified bands (sizes ranged from 210 bp to 3000 bp), and a significant amount of genetic polymorphism was identified across the six cultivars (Table 2).

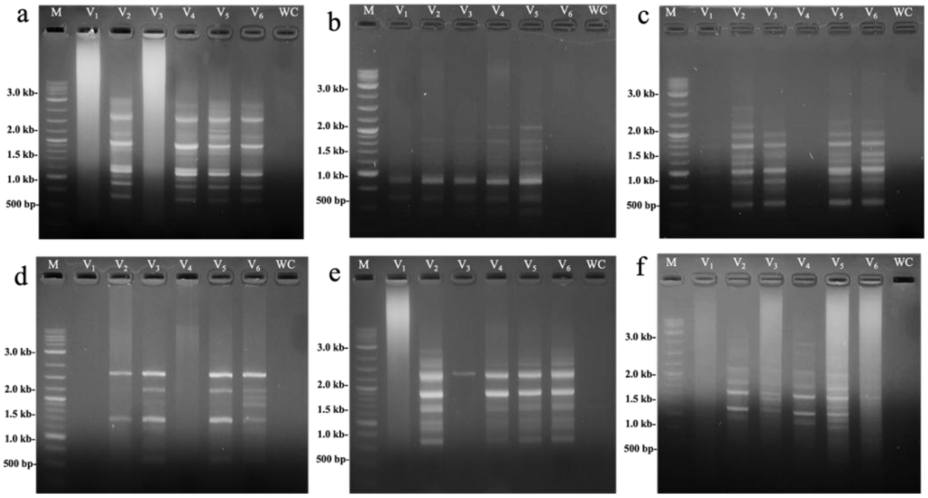

Fig. 1. RAPD analysis of six foxtail millet varieties: RAPD profiles showed distinct and polymorphic amplification patterns among the varieties, demonstrating considerable genetic diversity within the studied varieties. Lane 1-6, Amplified DNA of: (a) V_1 (BARI Kaon-1), (b) V_2 (BARI Kaon-2), (c) V_3 (BARI Kaon-3), (d) V_4 (BARI Kaon-4), (e) V_5 (Local Variety-Manikganj), and (f) V_6 (Local Variety-Bhola). Lane M= 1Kb plus DNA Ladder, Lane WC=Water Control).

Table 2. Levels of polymorphism within six Bangladeshi foxtail millet (*Setaria italica* L.) varieties.

Varieties	Total no. of bands	Total no. of polymorphic bands	Total no. of monomorphic bands	% of polymorphism exists in varieties	Average % of polymorphism exists in varieties
V_1 (BARI Kaon-1)	12	11	1	91.66	97.06
V_2 (BARI Kaon-2)	80	79		98.75	
V_3 (BARI Kaon-3)	38	37		97.36	
V_4 (BARI Kaon-4)	61	60		98.36	
V_5 (Local variety-Manikganj)	56	55		98.21	
V_6 (Local variety-Bhola)	52	51		98.07	

Moreover, the total no. of bands generated by individual RAPD marker for six varieties also counted, ranging from 6 to 48 bands (OPA-02: 48; OPA-03: 33; OPA-04: 43; OPA-06: 11; OPA-07: 10; OPA-08: 23; OPA-09: 27; OPA-10: 22; OPB-01: 6; OPB-04: 14; OPB-05: 38; OPB-06: 34; OPB-07: 32; OPB-08: 23 and OPB-10: 27). Further, the analysis of the scorable RAPD loci revealed an average of 8 loci per primer, resulting in 119 polymorphic loci out of a total of 120 loci. This resulted in an average polymorphism rate of 100% across the markers except for OPB-05 (90%) (Table 3). El-Hady et al. (2010) reported an average of 29.2 bands per primer, whereas the present study produced an average of 26.06 bands per primer using fifteen distinct RAPD primers. The relatively lower polymorphism rate was found in V_1 (91.66%) and V_3 (97.36%), while the higher polymorphism rate was observed in V_2 (98.75%), V_4 (98.36%), V_5 (98.21%), and V_6 (98.07%). According to Tabassum et al. (2013), 574 out of 584 bands generated in the RAPD trials were polymorphic, indicating a high degree of polymorphism with a percentage of polymorphic loci at 94.168. A wide range of polymorphisms has been identified in various crops using 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), 100% in mungbean (Sony et al. 2013), and 84.44% in cabbage (Saxena et al. 2011). Furthermore, each variety had one or more unique bands that were not present in others, which can serve as effective genetic markers for identifying these varieties. This indicates that these varieties are genetically diverse, which makes them suitable for breeding programs to develop improved varieties. The primers OPA-02, OPA-04, OPA-07, OPA-09, OPA-10, OPB-04, OPB-05, OPB-07, and OPB-10 successfully produced unique bands. A total of 42 unique bands were obtained by nine primers in six cultivars. The unique bands were specific to certain varieties and were effective markers for varietal differentiation.

Table 3. Levels of polymorphism within six foxtail millet (*Setaria italica* L.) varieties based on the marker.

Marker	DNA sequence 5'-3'	Total no. of RAPD loci	No. of polymorphic RAPD loci	Percentage (%) of polymorphism
OPA02	TGC CGA GCT G	14	14	100
OPA03	ATG CAG CCA C	8	8	100
OPA04	AAT CGG GCT G	11	11	100
OPA06	GGT CCC TGA C	4	4	100
OPA07	GAA ACG GGT G	4	4	100
OPA08	GTG ACG TAG G	7	7	100
OPA09	GGG TAA CGC C	9	9	100
OPA10	GTG ATC GCG T	7	7	100
OPB01	GTT TCG CTC C	2	2	100
OPB04	GGA CTG GAG T	10	10	100
OPB05	TGC GCC CTT C	10	9	90
OPB06	TGC TCT GCC C	9	9	100
OPB07	GGT GAC GCA G	11	11	100
OPB08	GTC CAC ACG G	7	7	100
OPB10	CTG CTG GGA C	7	7	100

The level of variability among six Bangladeshi foxtail millet varieties was estimated by a distance matrix based on RAPD bands (Table 4). Genetic distances among these varieties were calculated based on Nei's distance matrix (Nei 1972). This study revealed considerable genetic variability among the foxtail millet varieties. The genetic distances among the six varieties varied considerably, ranging from 0.1865 to 0.9146, as shown by Nei's pairwise genetic distance matrix. The greatest genetic distance (0.9146) was recorded between V_3 (BARI Kaon-3) and V_4 (BARI Kaon-4), while the smallest genetic distance (0.1865) was noted between V_5 (Local variety - Manikganj) and V_6 (Local variety - Bhola). The disparity between the maximum and minimum genetic distance values highlights a wide range of genetic variability among the six varieties of foxtail millet. In a similar study, Hazarika and Neog (2014) reported dissimilarities ranging from 0.08 to 0.43 among thirty accessions of Naga chili collected from three states in the North-East region of India.

Table 4. Summary of distance matrix of six foxtail millet (*Setaria italica* L.) varieties.

	V_1	V_2	V_3	V_4	V_5	V_6
V_1	0.0000					
V_2	0.6460	0.0000				
V_3	0.5675	0.5110	0.0000			
V_4	0.8166	0.4528	0.9146	0.0000		
V_5	0.6446	0.4775	0.6307	0.2220	0.0000	
V_6	0.7253	0.5512	0.7446	0.2486	0.1865	0.0000

Moreover, the genetic relationships among six foxtail millet varieties were visualized through a dendrogram constructed using the Neighbour-Joining method based on Nei's distance (Fig. 2). Cluster analysis, supported by genetic distances and the percentage of polymorphism in each variety, clearly distinguished all the varieties from one another. The dendrogram divided the varieties into two major clusters (C_1 and C_2), which included two sub-clusters and two sub-sub-clusters in the phylogenetic tree. In one major cluster, we found two varieties: V_1 (BARI Kaon-1) and V_3 (BARI Kaon-3). The other major cluster comprised V_2 (BARI Kaon-2), V_4 (BARI Kaon-4), the local variety Manikganj (V_5), and the local variety Bhola (V_6). The genotypes located in the same clusters were genetically more like one another, indicating a lower genetic distance that signifies a close relationship among the genotypes. For instance, the genetic distance between V_5 (local variety-Manikganj) and V_6 (local variety-Bhola) is 0.1865, suggesting a close relationship since both are local varieties. Similarly, V_2 (BARI Kaon-2) and V_4 (BARI Kaon-4), which were collected from the same sources, exhibited a lower genetic distance of 0.4528. In contrast, V_1 (BARI Kaon-1) and V_4 (BARI Kaon-4), despite being from similar sources (BARI-released varieties), showed a higher genetic distance of 0.8166. This indicates that the origins of these genotypes might be the same but adapted to different regions. Mokkaraj and Geethanjali (2016) categorized foxtail millet genotypes into four main clusters and ten sub-clusters using hierarchical cluster analysis based on quantitative features. However, the present study, identified only two main clusters, two sub-clusters, and two sub-sub-clusters. Akter (2020) reported a polymorphism rate of 78.05% among Indian foxtail millet varieties. In contrast, the present study, which evaluated four BARI-released varieties (BARI Kaon-1, BARI Kaon-2, BARI Kaon-3, and BARI Kaon-4) together with two local varieties from Manikganj and Bhola, revealed an substantially higher level of polymorphism (97.06%). Among six varieties, V_2 (BARI Kaon-2) exhibited the highest level of polymorphism at 98.75%, while V_1 (BARI Kaon-1) showed the lowest polymorphism at 91.66%. Additionally, the locally cultivated varieties V_5 and V_6 are genetically closely related, whereas V_3 and V_4 are the most distantly related in terms of their genetic distance matrix.

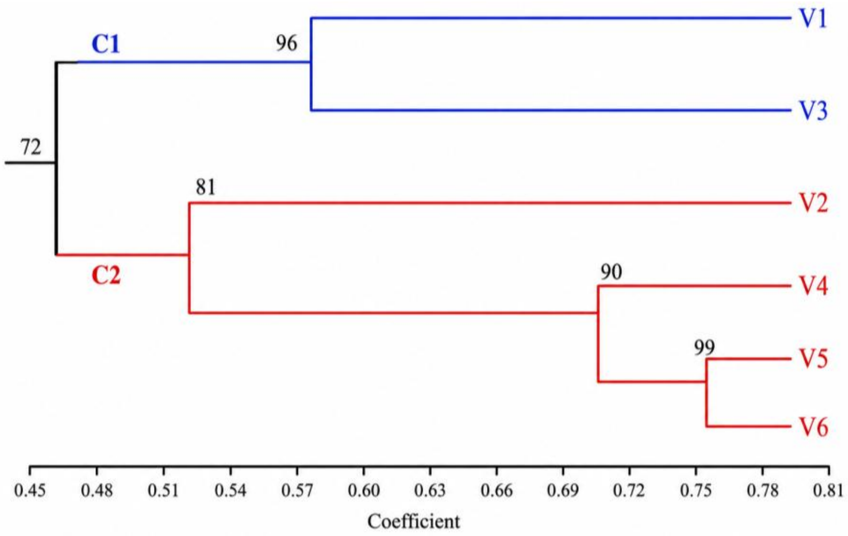

Fig. 2. Dendrogram illustrating the genetic relationships among six foxtail millet (*Setaria italica* L.) varieties based on RAPD marker data. The dendrogram was constructed using Nei's (1972) genetic distance matrix and the neighbour-joining (NJ) method. The analysis grouped the six varieties into distinct clusters, reflecting their genetic similarities and differences. V_1 (BARI Kaon-1), V_2 (BARI Kaon-2), V_3 (BARI Kaon-3), V_4 (BARI Kaon-4), V_5 (Local Variety–Manikganj), and V_6 (Local Variety–Bhola) exhibited varying degrees of genetic divergence, indicating the presence of considerable genetic diversity among the studied germplasms. The clustering pattern gives valuable information on genetic relationships and potential breeding value of these foxtail millet varieties.

In a nutshell, this is the first molecular level investigation in Bangladesh to assess genetic variability of foxtail millet using RAPD molecular markers. It is an important underutilized grain crop, which possesses resilience to adverse climatic condition, as well as the potential for sustainable agricultural system. Hence, it deserves more scientific attention in Bangladesh. The RAPD analysis revealed significant genetic variation among the six foxtail millet genotypes collected from various regions of the country. The obtained results from this study proves that the RAPD-based markers are effective and reliable tools for evaluating genetic variability, and genetic relationships. Additionally, the molecular characterization approach employed in this study was very informative and could be applied to other economically important crops. Genetic variability revealed by this research provides valuable material for future hybridization and crop improvement programs. Consequently, this pioneering research will improve our knowledge about foxtail millet genetics, which aid in developing better breeding strategies for sustainable agriculture and conservation of minor grain resources in Bangladesh.

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