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POLYMORPHISM STUDY IN BARLEY *(Hordeum vulgare)* GENOTYPES USING MICROSATELLITE (SSR) MARKERS

MANIRUZZAMAN[1], Z. A. TALUKDER[2], S. ROHMAN[3]
F. BEGUM[4] AND M. AMIRUZZAMAN[5]

Abstract

The experiment was conducted at the Molecular Breeding Lab of Plant Breeding Division, Bangladesh Agricultural Research Institute (BARl) to assess the inter and intra species diversity within the barley genotypes. Ten barley genotypes were used for polymorphism study through SSR markers. Among them, six markers showed distinct polymorphism within the barley genotypes. It was observed that the genotypes BB-1 and BB-3 were more diverged (0.255) compared to other genotypes. On the other hand, the genotypes BB-2, P-33, P-19, and P-25 were very much similar in their genetic level (83.00%) followed by BB-2, BB-4, BHL-19, P-19, BHL-18, BB-5, P-19, P-25, and P-33 (78.70%). The two dimensional graphical view of Principal Coordinate Analysis (PCO) showed the spatial distribution of the 10 barley genotypes along the two principal axes. The genotypes viz., BB-1 and BB-3 were found far away from centroid of the cluster and rest of the genotypes were placed around the centroid (Fig. 8). The genotypes that placed far away from the centriod were more genetically diverged compared to the genotypes placed near the centriod which were likely to be genetically more similar.

Keywords: Barley, SSR marker and diversity.

Introduction

Barley *(Hordeum vulgare* L.), a member of the grass family, is a major cereal grain. It is a self pollinating, diploid species with 14 chromosomes. Barley is a widely adaptable crop. It has a short growing season and is more tolerant to soil salinity than wheat and is also relatively drought tolerant. So it has the potentiality to meet the 2151century's challenge of climate change. For the rational use of genetic resources information on the genetic diversity within and among closely related crop varieties is essential. The development of molecular techniques for genetic analysis has led to a great increase in our knowledge of cereal genetics and our understanding of the structure and behaviour of cereal genomes. These molecular techniques, in particular the use of molecular markers, have been used to monitor DNA sequence variation in and among the species and create new sources of genetic variation by introducing new and favourable traits

[1]Senior Scientific Officer, [2,3&4]Scientific Officer, [5]Principal Scientific Officer, Plant Breeding Division, Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh.

from land races and related grass species. Improvement in marker detection systems and the techniques have enabled great advances in recent years. Microsatellite or simple sequence repeats (SSR) markers have been developed more recently for major crop plants and this marker system is predicted to lead to even more rapid advances in both marker development and implementation in breeding programmes. Microsatellites have been successfully "applied for detection of genetic diversity (Donini *et al.,* 1998; Korzun *et al.,* 2001), genome mapping (Korzun *et al.,*1997, Russell *et al.,* 1997), marker assisted selection of agronomically important traits (lena *et al.,* 2008) and genotype differentiation (Donini *et al.,* 1998; Virk *et al.,* 1999). SSR markers have many advantages for genetic studies over other markers. They are highly polymorphic, locus specific and abundant. Besides, they are distributed over the genome and require only small amounts of genomic DNA for analysis. Microsatellite markers have been developed in many crop species, such as soybean, wheat, maize, barley, rice, and potato. In cereals, they show a much higher level of polymorphism than other marker systems. Molecular genetic maps of crop species find a variety of uses not only in breeding but also in genomic research. Marker assays based on the polymerase chain reaction (PCR) offer a far more user-friendly marker system. However, microsatellites (di-or tri-nucleotide repeat sequences) also known as simple sequence repeats (SSRs) are highly reliable, co-dominant markers. Microsatellites are becoming more widely used for marker assisted breeding and variety identification due to their high level of polymorphism and ease of use. SSRs have recently become important genetic markers in a wide range of crop species including barley and wheat. They appear to be ubiquitous in higher organisms, although the frequency of microsatellites varies between species. They are abundant, dispersed throughout the genome, and show higher levels of polymorphism than other genetic markers. These features coupled with their ease of detection have made them useful molecular markers. Their potential for automation and their inheritance in a codominant manner are additional advantages when compared to other types of molecular markers. SSR loci are believed to evolve in a step-wise manner by the addition or subtraction of a single repeat. Up to 37 different alleles for the one SSR locus have been found in barley.

In Bangladesh, barley is a minor cereal crop and it is not widely grown over the country. Only Bangladesh Agricultural Research Institute (BARI) and few contract growers are cultivating it in a minor scale. At present, BARI is doing maintenance breeding of barley but molecular assessment is not done yet. Therefore, the study has designed to 1) develop and characterize microsatellite markers in barley, and 2) evaluate SSR markers for hybridity testing.

Materials and Method

Plant material

To study the molecular diversity, we used 10 barley genotypes and these were randomly selected from available germplasm (Table 1)

Table 1. List of barley genotypes.

SI No.	Barley genotypes
1	BB-l
2	BB-2
3	BB-3
4	BB-4
5	BB-5
6	BHL-18
7	BHL-19
8	P-19
9	P-25
10	P-33

Seedling raising

Seeds were grown in plastic pots. Then the pots were kept under the net house. After 15 to 20 days (3 or 4 leaf stage) the fresh leaf was used to DNA isolation.

Isolation of DNA and quantification of DNA concentration

Total DNA was isolated by CTAB method with slight modifications according to Maaβ and Klass (1995).After treatment with 10µg/ml RNase A for half an hour at 37°C, the DNA was purified with propanol. The purified DNA was dissolved in TE buffer and stored at -20°C and the concentration was determined fluorometrically (Nano drop).

Primers and PCR amplification

Eight SSR primer pairs were chosen (Table 2) to evaluate the polymorphism among the genotypes. PCR conditions were optimized. Here amplifications were performed in 20µl volumes containing 100ng genomic DNA, 2.5 mM dNTPs, 1.5mM $MgCI_2$, 10 micro mole each forward and reverse primers, 3U TaqDNA polymerase and l0X PCR buffer (Genei). Thermal cycling consisting of initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 1 min, annealing temperature 55°C for 1 min and 72°C for 1 min followed by a final extension at 72°C for 5 min. PCR products were stored at 4°C until use.

Table 2. SSR markers used for the determination of molecular variations.

SL No.	Primer	Sequence of the Primer	Chrom No.	Length (bp)	PIC Value
01	Bmac0040	Forward: AGCCCGA TCAGATIT ACG Reverse: TTCTCCCTTTGGTCCTIG	6H	36	0.894
2	Bmag021 1	Forward: ATICATCGATCTTGTATTAGTCC Reverse: ACATCATGTCGATCAAAGC	IH	42	0.825
3	Bmac0134	Forward: CCAACTGAGTCGA TCTCG Reverse: CTICGTIGCTICTCT ACCTT	2H	38	0.761
4	Bmac0181	Forward: ATAGATCACCAAGTGAACCAC Reverse: GGTTATCACTGAGGCAAATAC	4H	42	0.747
5	Bmag0131	Forward: TTICAGAAACGGAGTITTG Reverse: CCTCCACACAAAAAA TCC	3H	37	0.866
6	Bmac0156	Forward: AACCGAA TGTATTCCTCTGT A Reverse: GCCAAACAACT ATCGTGT AC	7H	41	0.890
7	EBmac0713	Forward: GGTAAAACATTTCCCTCGT Reverse: TAGAGATCACTCTCTTCTGTGC	7H	41	0.904
8	Bmag0135	Forward: ACGAAAGAGTTACAACGGATA Reverse: GTTTACCACAGATCTACAGGTG	7H	43	0.888

Polyacrylamide Gel Electrophoresis (PAGE)

Preparation of gel

Polyacrylamide gel electrophoresis or PAGE is generally used to resolve DNA in the low molecular size range of 50-100 basepair (bp) and detect up to single bp differences. The following chemicals were used to prepare two plates of PAGE gel (Table 3).

Table 3. Reagents and their amount for the preparation of PAGE gel plate.

SI. No.	Reagent	Amount
2	Sterile distilledwater	13.78ml
2	10xTBEbuffer solution	2ml
3	40% Acrylamide-BisAcrylamide	4ml
4	1O%Ammoniumpersulfate (APS)	200 µl
5	TEMED	17 µl
	Total	20ml

Visualization of the gel

Staining solution was carefully prepared by adding 65 µl ethidium bromides in 500 ml sterile distilled water. The staining solution was used for staining of 2 gels. The gels were stained for 30- 35 minutes in dark and were documented using UVPRO Alpha Innotech gel documentation unit.

Data analysis

Molecular weight for each amplified allele was measured in base pair using Alpha-Ease FC 5.0 software. The allele frequency data from Power Marker version 3.25 (Liu and Muse, 2005) was used to export the data in binary format (allele presence="I" and allele absence = "0") for analysis with NTSYS-pc version 2.2 (Rohlf, 2002). Gene diversity referred to as Polymorphic information content (PIC) values were calculated with the following formula (Anderson *et al.,* 1993):

$$PICi = 1 - \sum_{f=1}^{n} (Pij)^2$$

Where, n is the number of marker alleles for marker i and P_{ij} is the frequency of the j^{th} allele for marker i.

The summary statistics including the number of alleles per locus, major allele frequency, gene diversity, polymorphism information content (PIC) values were determined using Power Marker version 3.25 (Liu and Muse, 2005). A similarity matrix was calculated with the Simqual subprogram using the Dice coefficient followed by cluster analysis with the SAl IN subprogram using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Clustering method as implemented in NTSYS-pc was used to construct a dendrograms howing relationship among the genotypes. The similarity matrix was also used for principal coordinate analysis (PCA) with the D Center, Output, and MXPlot subprograms in computer program Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) 12.

$GD=I-d_{xy}/ (d_x+d_y-d_{xy})$,where GD = Genetic distance between two genotypes, d_{xy} = total number of common loci (bands) in two genotypes, d_x= total number of loci (bands) in genotype 1 and d_y= total numbers of loci (bands) in genotype 2.

Results and Discussion

Eight primers were used to analyze the molecular diversity of 10 barley genotypes. Among them, six (Bmac0156, Bmac0040, Bmag0211, Bmac0134, Bmac0181, and Bmag0131) primers produced interpretable polymorphism based on co-dominant marker system bands (Fig. 2 to Fig. 4). The other, two markers (EBmac0713 and Bmag0135) showed monomorphic pattern and hence were not

included in further analysis. Amplified micro satellite loci were analyzed for polymorphism using polyacrylamide gel electrophoresis. The results of polymorphism for six SSR markers are shown in Fig. 1 to Fig. 5. Number of alleles, range of allele (bp) and gene diversity (GD) found in 10 barley genotypes using six SSR markers are shown in (Table 4). The total of 47 alleles was detected by 8 SSR markers among 10 barley genotypes with an average of7.8 alleles per-microsatellite/genotypes locus. We observed the highest number of alleles per locus/genotype using two SSR primers set Bmac0040 and Bmac0134 showing 9 alleles (Table 4). The lowest allele number per locus among the homologous chromosomes was observed using SSR primer set Bmac0156, showing a total of with an average of 0.6 alleles per genotype (Table 4). However, Ivandic *et al.* (2000) also found similar findings (5.5 alleles per locus) from wild barley (Fertile Crescent).

It is important to note that the total number of alleles reported in diversity studies is usually proportional to sample size, and some differences were observed which might be due to sampling differences. However, another factor influencing the number of alleles is the use of di-nucleotide repeat SSRs, which can produce large number of alleles. The highest allele size difference was found in Bmac040 followed by Bmac0l81 and the lowest was in Bmac0l56. According to Nei's (1973), the highest level of gene diversity value (0.8800) was observed in loci Bmac0040 and Bmac0134 and the lowest gene diversity value (0.7600) was observed in loci Bmac0156 with a mean diversity of 0.7773. It was observed that marker detecting the lower number of alleles showed lower gene diversity than those detected the higher number of alleles showed higher gene diversity. This result is consistent with previous work done by Herrera *et al.* (2008).

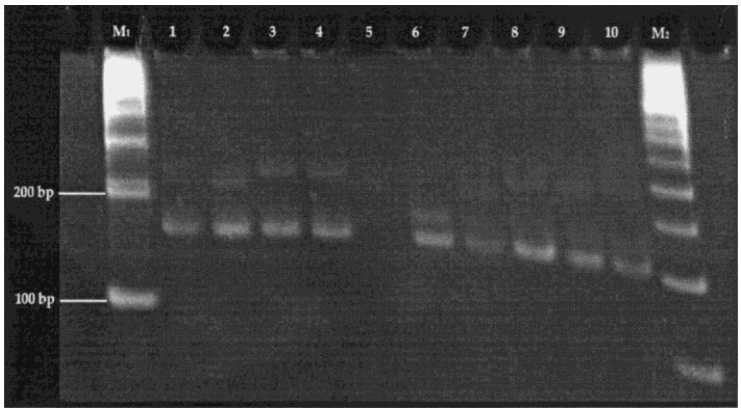

Fig. 1. DNA profile of 10 different barley genotypes with SSR marker mac0040. Accession numbers BB-4, BB-2, BHL-19, BHL-18, BB-l, BB-3, BB-5, P-19, P-25, and P-33 were arranged sequentially from 1-10, M: Ladder DNA.

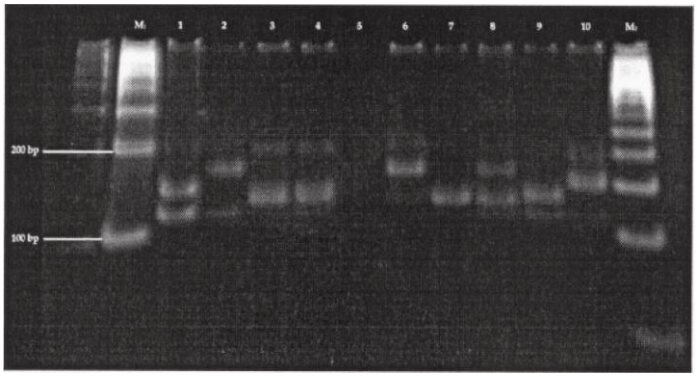

Fig. 2. DNA profile of 10 different barley genotypes with SSR marker Bmac0134. Accession numbers BB-4, BB-2, BHL-19, BHL-18, BB-1, BB-3, BB-5, P-19, P-25, and P-33 were arranged sequentially from 1-10, M: Ladder DNA.

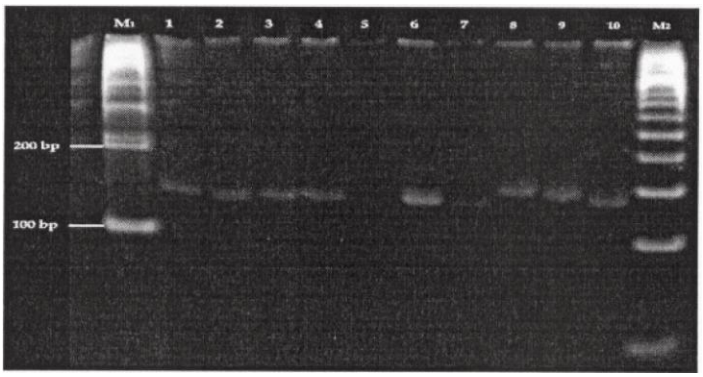

Fig. 3. DNA profile of 10 different barley genotypes with SSR marker Bmag0131. Accession numbers BB-4, BB-2, BHL-19, BHL-18, BB-1, BB-3, BB-5, P-19, P-25, and 1>-33were arranged sequentially from 1-10, M: Ladder DNA.

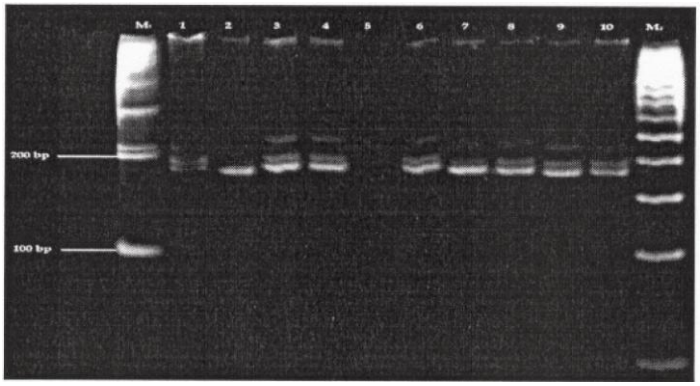

Fig. 4. DNA profile of 10 different barley genotypes with SSR marker Bmac0181. Accession numbers BB-4, BB-2, BHL-19, BHL-18, BB-1, BB-3, BB-5, P-19, P-25, and P-33 were arranged sequentially from 1-10, M: Ladder DNA.

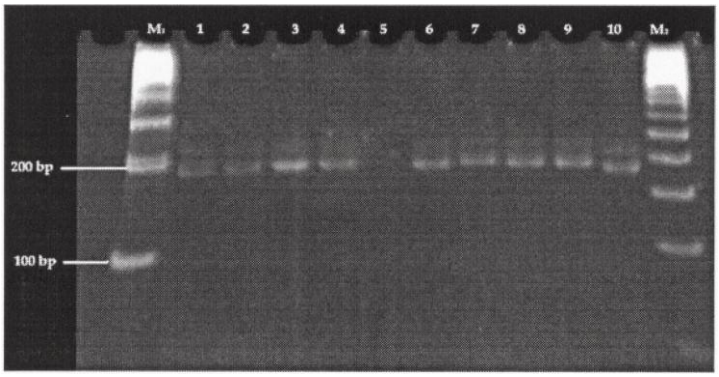

Fig. 5. DNA profile of 10 different barley genotypes with SSR marker mag0211. Accession numbers BB-4, BB-2, BHL-19, BHL-18, BB-1, BB-3, BB-5, P-19, P-25, and P-33 were arranged sequentially from 1-10, M: Ladder DNA.

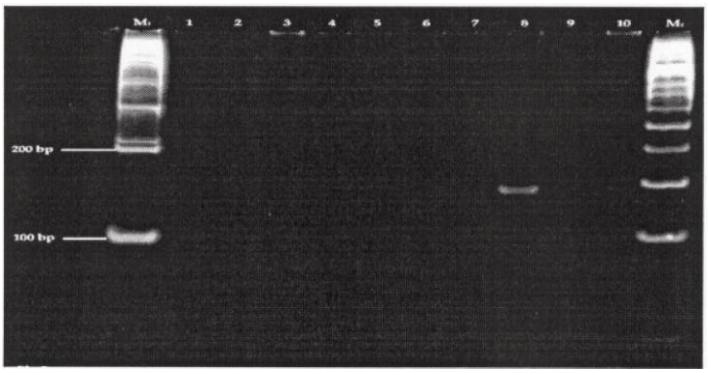

Fig. 6: DNA profile of 10 different barley genotypes with SSR marker Bmag0135. Accession. numbers BB-4, BB-2, BHL-19, BHL-18, BB-1, BB-3, BB-5, P-19, P-25, and P-33 were arranged sequentially from 1-10, M: Ladder DNA.

Table 4. Number of alleles, range of allele (bp) and gene diversity (GD) found in 10 barley genotypes for 6 SSR markers.

SI No.	Markers	Chroa No.	Repeat type	Allele no.	Allele size (bp)	Gene diversity
1.	Bmac0040	6H (143cM)	(AC)20	9	236	0.8800
2.	Bmag02ll	IH (61.6cM)	(CT) 16	8	174	0.8600
3.	Bmac0134	2H (15.1cM)	(AC)28	9	148	0.8800
4.	Bmac0181	4H (45.1cM)	AC)20	7	177	0.8200
5.	Bmag0131	3H (52cM)	(AG)16G(AG)15	8	149	0.8600
6.	Bmac0156	7H (149.5cM)	(AC)22(AT)5	6	139	0.7600
	Mean			7.8		0.8433

The frequency of the most common allele at each locus ranged from 20.00% (Bmac0040, Bmag0211, Bmac0134 and Bmag0131) to 40.00% (Bmac0156). On an average, 25.00% of the 10 barley genotypes shared common major allele at any given locus (Table 5). The polymorphic information content (PIC) values ranged from 0.7300 to 0.8680 with an average of 0.8253. The highest PIC value (0.8680) was obtained for Bmac0040 and Bmac0134 followed by Bmag0211 and Bmag0131 (0.8442), Bmac0181 (0.7978) (Table 5). The lowest PIC value (0.7300) was obtained for Bmac0156 (Table 5). PIC value revealed that Bmac0040 and BmacO134 were considered as the best marker for 10 barley genotypes followed by Bmag0211 and BmagO131. Bmac0156 could be considered as least powerful marker.

Table 5. Data on no. of observations, major alleles frequencies and Polymorphism information content (PIC) found among 10 barley genotypes.

Sl. No	Locus	No. of observations	Major allele PIC frequencies (%)	PIC	Mean PIC
1	Bmac0040	10	0.2000	0.8680	
2	Bmag0211	10	0.2000	0.8442	
3	Bmac0134	10	0.2000	0.8680	0.8253
4	Bmac0181	10	0.3000	0.7978	
5	Bmag0131	10	0.2000	0.8442	
6	Bmac0156	10	0.4000	0.7300	

Genetic distance-based analysis

UPGMA similarity matrix

From the similarity index (Table 6), the pair-wise genetic similarity coefficients indicated that the genotype BB-2, P-33, P-19, and P-25 were very much similar in their genetic level (83.00%) (Table 6) followed by BB-2, BB-4, BHL-19, P-19, BHL-18, BB-5, P-19, P-25, and P-33 (78.70%). So, in crop improvement programme, these genetically similar parents could not be chosen in the future crossing programme to create genetic variability. On the other hand, the lowest similarity (74.5%) was found in almost all genotypes (Table 6). It means, BB-l showed diversity (25.5%) with BB-2, BB-3, BB-4, BB-5, BHL-18, BHL-19, P-19, P-25, and P-33. Furthermore, BB-2 showed diversity (25.5%) with BB-3, BB-5, BHL-18, and P-25. BB-3 showed diversity (25.5%) with BB-4, BB-5, BHL-18, BHL-19, P-19, P-25, and P-33. Besides, BB-4 showed diversity (25.5%) with BB-5, BHL-19, P-19, P- 25 and P-33 (Table 6). In case of BB-5, it

showed diversity (25.5%) only with BHL-18 (Table 6). So, in crop improvement, by considering this molecular diversity as well as phenotypic diversity study, we can choose the best variety or inbred lines.

A dendrogram based on UPGMA cluster analysis (Fig. 7) of the SSR data showed two genotypes BB-l and BB-3 formed single cluster. Other genotypes formed two distinct clusters. Here BB-2 and P-33 belongs to the same sub cluster and P-19, P-25 belong to the same sub cluster but these four genotypes formed the same cluster. In addition, BB-5 and BHL-19 are on the same cluster and BB-4 and BHL-18 are on the same cluster group. Since BB-I and BB-3 formed two individual clusters, hence they are more diverged parent than the other population.

So, future breeding programme for crop improvement, we can choose these genetically diverged parents for crossing programme to create genetic variability and transgressive segregants.

Principal coordinate analysis

The two dimensional graphical view of Principal Coordinate Analysis (PCa) showed the spatial distribution of the 10 barley genotypes along the two principal axes. The genotypes viz., BB-I and BB-3 were found far away from centroid of the cluster and rest of the genotypes were placed around the centroid (Fig. 8). The genotypes placed far away from the centriod were more genetically diverged compared to the genotypes placed near the centriod, which were likely to be genetically more similar. However, centriod may be deemed as the vector representing the middle point of the cluster which contained at least one number for each variable. The connecting line between the each genotype and the centriod represented eigen vectors for the respective genotypes. The three dimensional (3D) graphical view of Principal Coordinate Analysis (PCa) showed the spatial distribution of the 10 barley genotypes along the three principal axes. Principal coordinate analysis also collaborated with the result from UPGMA cluster analysis. The 3D diagram (Fig. 9) helped visualize four major clusters and showed that two barley genotypes (BB-I and BB-3) were far away from the other genotypes.

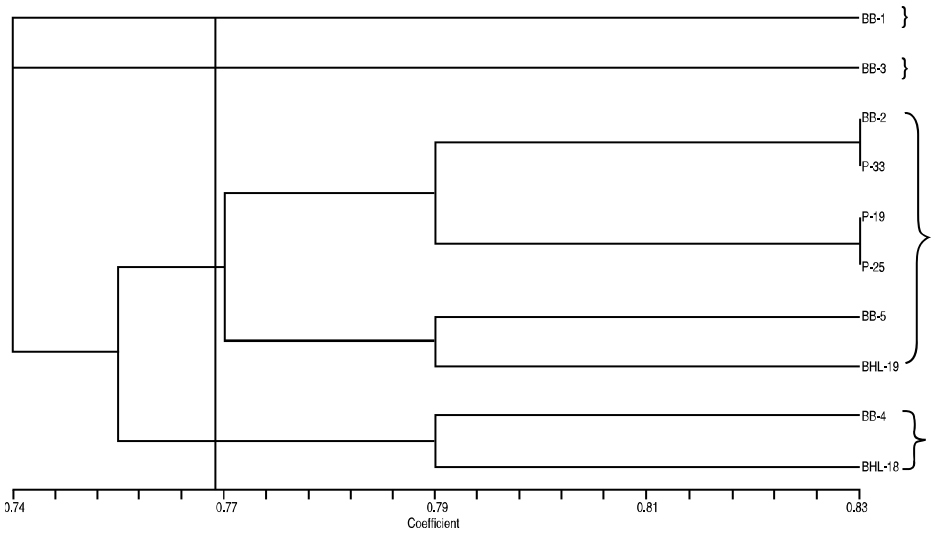

Fig. 7. A UPGMA cluster dendrogram showing the genetic relationships among barley genotypes based on the alleles detected by 6 SSR markers.

Table 6. Genetic similarity index of micro satellite patterns of 10 barley genotypes using 10 SSR markers.

Rows\Cols	BB-1	BB-2	BB-3	BB-4	BB-5	BHL-18	BHL-19	P-19	P-25	P-33
BB-1	1.000									
BB-2	0.745	1.000								
BB-3	0.745	0.745	1.000							
BB-4	0.745	0.787	0.745	1.000						
BB-5	0.745	0.745	0.745	0.745	1.000					
BHL-18	0.745	0.745	0.745	0.787	0.745	1.000				
BHL-19	0.745	0.787	0.745	0.745	0.787	0.787	1.000			
P-19	0.745	0.787	0.745	0.745	0.787	0.745	0.745	1.000		
P-25	0.745	0.745	0.745	0.745	0.787	0.787	0.745	0.830	1.000	
P-33	0.745	0.830	0.745	0.745	0.787	0.745	0.745	0.830	0.787	1.000

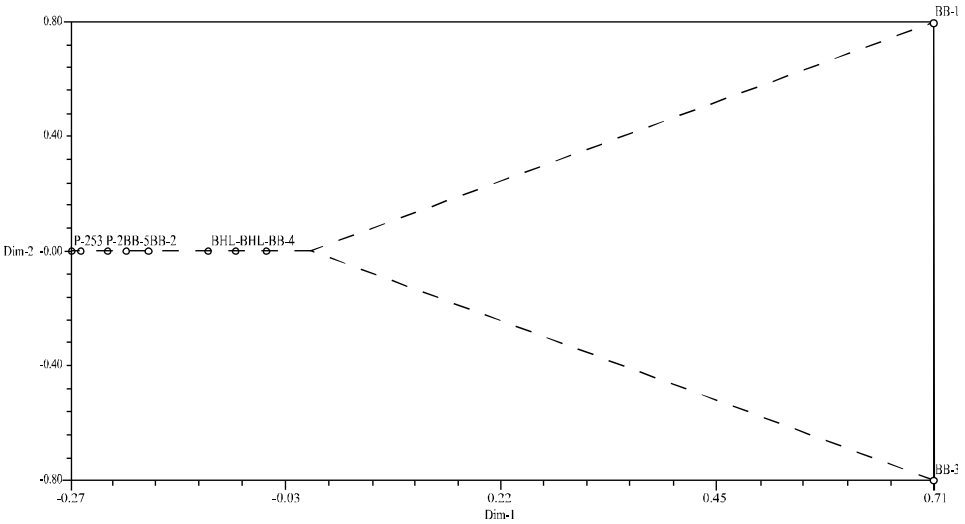

Fig. 8. Two-dimensional view of principal coordinate analysis (PCa) with 6 SSR markers over 10 barley genotypes.

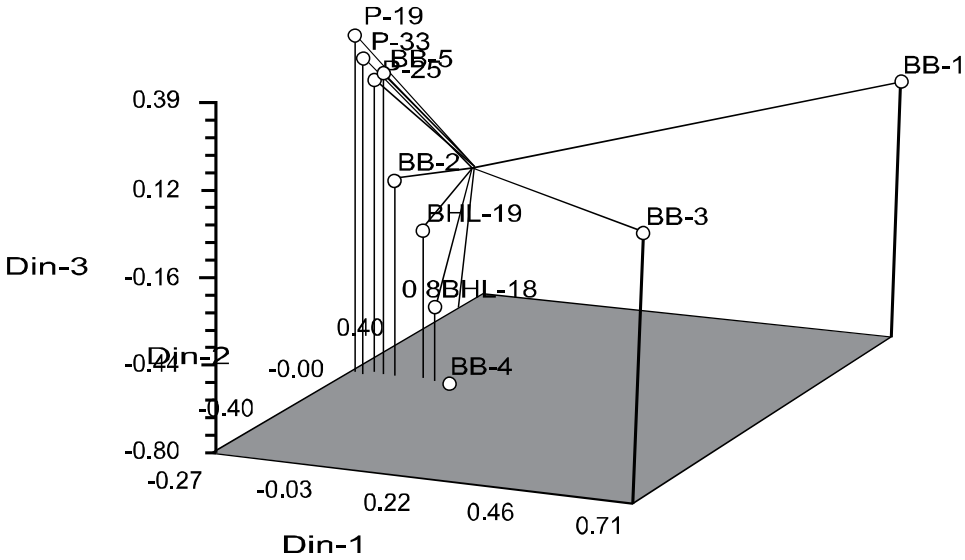

Fig. 9. Three- dimensional view of principal coordinate analysis (PCO) with 6 SSR markers over 10 Barley genotypes.

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