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Genetic Diversity Assessment in Several Barley (*Hordeum vulgare* L.) Cultivars Using Microsatellite Markers

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Abstract

In the present study, genetic diversity in seven cultivars of cultivated barley (*Hordeum vulgare*) populations was evaluated using 10 microsatellite markers. Genomic DNA was extracted from fresh leaves and amplification reactions were done by PCR. The amplification products were separated on 6% denaturing polyacrylamide gels containing 7M urea and visualized via silver staining method. High level of polymorphism was observed among populations. Polymorphic bands ranged from 100 to 300 bp. Altogether 65 alleles were observed among all genotypes, with an average of 9.2 alleles per locus for all loci. Polymorphic information content (PIC) ranged from 0.80 to 0.88 with an average of 0.84. 'Sahand' populations showed the lowest mean of gene diversity whereas the highest mean of heterozygosity observed in Rayhan populations that can prepare a powerful resource of genetic diversity for breeding programs. The genotypes were clustered using unweight pair-group method on arithmetic average by POPGEN32 software. The dendrogram discriminated all the genotypes in several groups. The results showed that SSR markers have a high ability to reveal most of the information in a single locus and can be used for genetic analysis in molecular levels determination of genetic similarity and clustering barley cultivars.

Keywords: barley, genetic diversity, microsatellite markers, SSR

Introduction

Utilization of germplasm resources and efficient conservation need ample knowledge regarding the amount of genetic variation in germplasm arrays and genetic relationships between genotypes. Selection of parental combinations that will maximize gain from selection and maintain genetic diversity depends on the information about the amount of genetic variation present, and the location of the genetic determinants of diversity (Matus and Hayes, 2002). Barley, *Hordeum vulgare* L., is one of the major crops in the world that is cultivated in all temperate areas and is an economically important cereal ranking fourth in world crop production (Hayes *et al.*, 2003). Barley is considered a model species for genetic analysis. Molecular markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. Many types of molecular markers have been used to characterize germplasm, with each method differing in principle, application, type and amount of polymorphism detected, cost and time requirement. Microsatellite markers are tandem, 1 to 5, nucleotide repeats found in eukaryotic genomes. SSRs are co dominant, abundant, and informative and their detection can be automated. This makes them excellent molecular marker system for many types of genetic analyses, including linkage mapping, germplasm surveys, and phylogenetic studies (Liu *et al.*, 1996). Most SSRs are highly reproducible and demonstrate a high degree of allelic variation. Nowadays the effectiveness and informative value of microsatellite markers in genetic studies has been demonstrated for all the major cereals (Sjakste *et al.*, 2003). Microsatellites in barley were also used to study genetic diversity and trace the development of germplasm (Macaulay *et al.*, 2001; Struss and Plieske, 1998). Microsatellite maps for all seven barley chromosomes are now available for the public (Saghai-Maroof *et al.,* 1994; Ramsay *et al.,* 2000).

The objectives of this study were to investigate SSR polymorphism and genetic diversity of 10 microsatellite loci in a set of 7 barley cultivars and to determine the amount of genetic distance and similarity among populations.

Materials and methods

Seven cultivars of Iranian cultivated barely ('Karoon', 'Valfajr', 'Makooee', 'Reyhan', 'Cb74-2', 'Sahand' and 'Zarjo') were selected for this study. All of these samples were obtained from Research Station of Agricultural and Natural Resources, Khorasan Razavi, Iran.

DNA extraction

Genomic DNA was extracted from a bulk sampling of a minimum of 20 individuals for each variety using McPherson and Moller (2001) method. Equivalent amounts of DNA from 20 individual plants were pooled as 3 bulked DNA samples for PCR analyses.

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SSR assay

Ten primer pairs (from Metabion International Inc, Germany) were either derived from sequences published previously or designed based on sequences containing tandem repeats from the EMBL and GenBank databases (Tab. 1). PCR amplifications were performed in a total volume of 20 µl, containing 25 ng of barley genomic DNA, 1x PCR buffer, 0.5 mM both primers, 0.2 mM dNTPs, 2.25 to 3 mM $MgCl_2$ (based on Tab. 1) and 1 unit of *Taq* polymerase. Amplifications were performed in an Eppendorf thermocycler. PCR amplifications were hot-started at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 50 s, annealing according to Tab. 1 for 45 s and extension at 72°C for 45 s. The final extension was done at 72°C for 5 min.

Gel and data analysis

The amplification products were separated on 1.7% agarose gel for initial analysis. The 3 primers (Bmac0032, Bmac0223 and Hvm27) which could not produce the product of interest, removed and experiments were continued using other 7 primers. The amplification products were separated on 6% denaturing polyacrylamide gels and visualized via the silver staining method (Bassam *et al.,* 1991). The resulting gels were scored manually. A similarity matrix using Nei coefficient was generated and the genotypes were clustered using the unweighted pair-group method on arithmetic average (UPGMA) by POPGEN32 software. To measure the informativeness of each SSR, the polymorphism information content (PIC), was calculated using the following formula: PIC = $1-\Sigma f_i^2$ where *fi* is the frequency of the *i* SSR allele (Smith *et al.*, 2000). Shannon information index were calculated for different loci for each population using the following formula:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

Where S is the number of alleles and p_i is the relative abundance of each allele (Hedrick, 1999a).

Results and discussion

Polymerase chain reaction products were electrophoresed on 1.7% agarose gel for primary evaluation. The whole primers except Bmac0032, Bmac0223 and HVM27 conducted to products of interest; therefore, the experiments were continued using 7 primers. High levels of polymorphism using these 7 primers were observed after separating PCR products on 6% polyacrylamide gel. The length of amplified DNA fragments, including microsatellites, ranged from 100 to 300 base pairs. Altogether 65 polymorphic alleles were observed among all genotypes, with an average of 9.2 allele per locus for all loci. At least one allele was observed per population. The number of alleles varied from 7 to 13 alleles per locus with an average of 9.28 alleles per locus. Maximum effective alleles (8 alleles) were observed in 'Reyhan' and 'Cb74-2' populations. The rest of the populations except 'Zarjo' and 'Valfajr' had one effective allele. The number of effective alleles among population at different loci ranged from 8.5 (Bmac0134) to 5.12 (Bmac0173) with an average of 6.65 effective allele per locus (Fig. 1).

In this study null alleles were detected at Bmac0173 and HVM7 loci in 'Reyhan' and 'Sahand' cultivars, respectively. The level of polymorphism observed in number of alleles per locus was high and comparable for all polymorphic loci. 'Zarjo' and 'Valfajr' showed the highest (100%) level of polymorphism, but 'Sahand' populations had a polymorphism level of 71.43% (Tab. 2). PIC values in this study were quite high and ranged from 0.80 (Bmac0173) to 0.88 (Bmac0134) with an average of 0.84 for SSR markers (Tab. 2). Maximum (0.8006) and minimum (0.5998) mean heterozygosity were observed at 'Reyhan' and 'Sahand' population respectively (Tab. 3). Thus, concerning the polymorphic information content and heterozygosity, 'Sahand' and 'Reyhan' cultivars were the most uniform and the most diverse populations respectively. 'Reyhan' population also showed the highest mean of gene (allele) diversity that indicated high level of heterozygosity in this cultivar.

Tab. 1. Polymerase chain reaction primers and their characteristics

Primer		Sequence 5′→3′	MW (g/mol)	Tm (C°)	Ta (C°)	$MgCl_2$ (MM)
HVCMA	F	GCCTCGGTTTGGACATATAAAG	6774	58.4	55	3
	R	GTAAAGCAAATGTTGAGCAACG	6816	56.5	55	
Bmac0134	F	C C A A C T G A G T C G A T C T C G	5460	56.0	53.1	2.5
	R	CTTCGTTGCTTCTCTACCTT	5976	55.3	53.1	
Bmac0173	F	C A T T T T T G T T G G T G A C G G	5552	51.4	50	3
	R	ATAATGGCGGGAGAGACA	5622	53.7	50	
HVM9	F	C T T C G A C A C C A T C A C C C A G	5678	58.8	52.5	2.3
	R	ACCAAAATCGCATCGAACAT	6063	53.2	52.5	
HVM7	F	ATGTAGCGGAAAAAATACCATCAT	7378	55.9	58.3	2.25
	R	CCTAGCTAGTTCGTGAGCTACCTC	7280	64.4	58.3	
HVM4	F	AGAGCAACTACCAGTCCAATGGCA	7340	62.7	67.2	3
	R	GTCGAAGGAGAAGCGGGCCCTGGTA	7797	69.5	67.2	
HVM3	F	ACACCTTCCCAGGACAATCCATTG	7242	62.7	61.2	3
	R	AGCACGCAGAGCACCGAAAAAGTC	7374	64.4	61.2	

Tab. 2. Polymorphism values and mean of PIC per population

Population	Polymorphic loci	Monomorphic loci	Polymorphism (%)	PIC
'Karoon'	18	3	85.71	0.6467
'Sahand'	15	6	71.43	0.5998
'Makooee'	18	3	85.71	0.6468
'Reyhan'	18	3	85.71	0.8006
'Cb74-2'	18	3	85.71	0.6544
'Valfajr'	18	0	100	0.7540
'Zarjo'	21	0	100	0.7593

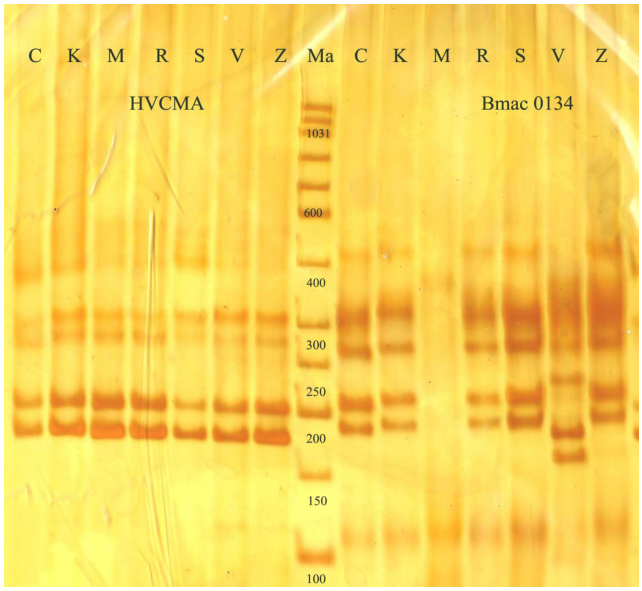

Fig. 1. SSR bands of various cultivars of Barley on 6% denaturating polyacrylamide gel. (Capital letters = first letter of a cultivar name)

The genetic distance (*D*) among genotypes was estimated based on the proportion of shared alleles. A dendrogram showing the genetic relationships between genotypes was constructed to express the results of cluster analysis based on data obtained by SSR amplification products. The dendrogram discriminated all the cultivars and clustered them separately in 7 groups (Fig. 2). The smallest genetic distance was observed between 'Zarjo' and 'Valfajr' cultivars. The maximum genetic distance observed between 'Sahand' and 'Makooee' populations. Shannon information index and Polymorphic information data (PIC) were calculated for different loci for each population (Tab. 3).

In this study, genomic DNA was extracted from a bulk sample of each variety. The advantages and inconvenience of the bulk analysis have been discussed by Michelmore *et al.* (1991) and Loarce *et al.* (1996). Bulk analyses are economic and rapid, and it is possible to estimate the genetic variability between accessions, whereas it is not possible to obtain information about the genetic variability within the accessions (Fernandez *et al.*, 2002). The number of individual plants bulked for the cultivars is an important experimental factor whether the bulked analysis revealed the genetic relationship between the cultivars. Yang and Quiros (1993) found that the bulked samples with 10, 20, 30, 40 and 50 individuals had the same banding pattern. Bustos *et al.* (1998) also found that bulks of 10 to 20 individuals resulted in the same RAPD profiles. In this study, we used a minimum of 20 individuals for representing each barley variety.

Altogether 65 polymorphic alleles were observed among all genotypes, with an average of 9.2 allele per locus for all loci. Different alleles represented different sizes of the amplification products (Sjakste *et al.*, 2003). The number of alleles ranged from 7 (HVM7 and HVCMA) to 13 (HVM3) alleles per locus. These results were in agreement with the results of Kraic *et al.* (2002). Maximum effective alleles (8 alleles) were observed in 'Reyhan' and 'Cb74-2' populations. The rest of populations except 'Zarjo' and 'Valfajr' had one effective allele. In most cases, each primer pair amplified only one microsatellite allele. This indicates homozygous states in the microsatellite locus but the presence of two or more alleles in some genotypes were observed. These genotypes were regarded as heterozygous at a given locus. Other explanations for this are the presence of two or more sister lines (microsatellite phenotypes), or the presence of an impurity, *i.e.* non-related genotype(s) in the bulked DNA sample (Kraic *et al.*, 2002). The number of effective alleles among population at different loci ranged from 8.5 (Bmac0134) to 5.12 (Bmac0173) with an average of 6.65 effective allele per locus. When the whole alleles have equal frequency, the number of effective alleles will be equal to $1/_F$ where F is the proportion of homozygotes, therefore, the proportion of homozygotes will be in inverse ratio to the number of effective alleles per locus in population. Since the alleles do not have equal frequency, the numbers of effective alleles are smaller than real alleles (Valdes *et al.*, 1993).

In this study null alleles were detected at Bmac0173 and HVM7 loci in 'Reyhan' and 'Sahand' cultivars respectively. We also note that from the perspective of the laboratory investigation the pseudo-death of an allele or locus can occur at any stage in the life cycle due to single (or multiple) nucleotide substitutions, insertions or deletions which occur in flanking regions and prevent primer binding. This process results in null alleles (Callan *et al.*, 1993), which may in time become fixed in the population (Chambers and MacAvoy, 2000). It is now well established that the predominant mutation mechanism in microsatellite tracts is 'slipped-strand mispairing' (Sia *et*

Tab. 3. Shannon information index and heterozygosity values of barley cultivars

Population	'Karoon'	'Valfajr'	'Makooee'	'Reyhan'	'Sahand'	'Cb74-2'	'Zarjo'
$H_{(mean)}$	0.6467	0.7540	0.6468	0.8006	0.5998	0.6544	0.7593
$I_{(mean)}$	1.2826	1.5350	1.2437	1.7861	1.1484	1.3462	1.5099

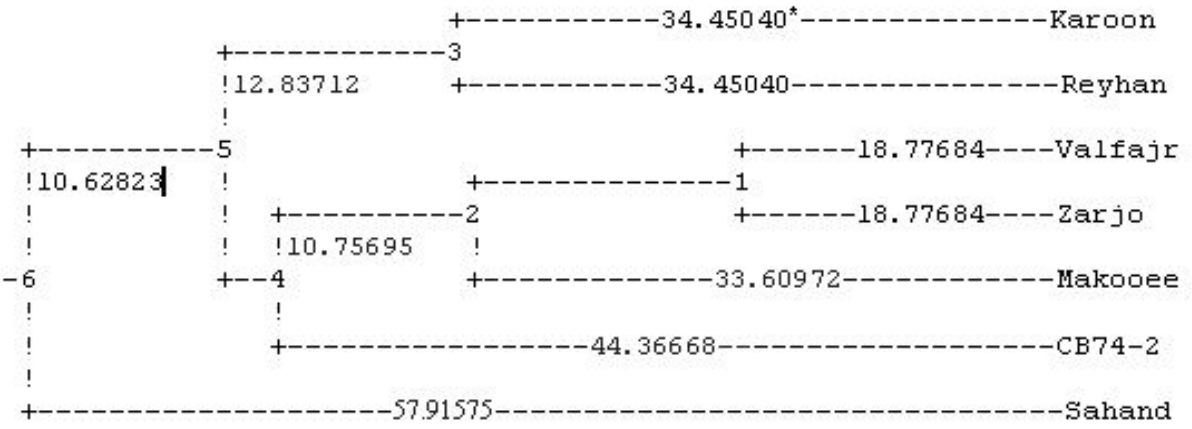

Fig. 2. Dendrogram based Nei's (1972) genetic distance among populations of barley by Popgen 32. *Distances between locations

al., 1997). This process has been well described by Eisen (1999). When slipped-strand mispairing occurs within a microsatellite array during DNA synthesis, it can result in the gain or loss of one, or more, repeat units depending on whether the newly synthesized DNA chain loops out or the template chain loops out respectively.

Since the heterozygotes contain various alleles, their frequency is important as a diversity indicator. Heterozygosity is the most common scale for evaluating genetic diversity in a population (Hedrick, 1999b). Weir (1996) suggested the gene diversity (D) term instead of heterozygosity. Both scales are reported for a locus or average of several loci. However heterozygosity is the most common scale for evaluating allelic variations or effectiveness of a molecular marker (Nei, 1978). It is noticeable that the heterozygosity scales are not sensitive to diversity increase because the extremity of heterozygosity for every number of alleles is equal. This limitation complicates separation of populations using highly variable loci such as microsatellites (with heterozygosity of 0.8 or more). Shannon information index was calculated for different loci for each population:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

The comparison of heterozygosity values as an inter-population diversity scale is not accurate for high polymorphic markers such as microsatellites (with heterozygosity of 0.8 or higher) and the differences in such level may not gain exact information. Whereas the maximum value of Shannon's information index is equal to Ln (s), it may be useful index for measure of diversity of highly variable loci (Hedrick, 1999a). The results of Shannon information index in comparison with heterozygosity or the polymorphism information content (PIC) values indicate that Shannon information index was similar to heterozygosity values and both indices corroborate their results (Tab. 3).

Senior *et al.* (1998) reported that PIC is synonymous with the term "gene diversity" as described by Weir (1996).

The dendrogram discriminated all the cultivars and clustered them separately in 7 groups (Fig. 2). The smallest genetic distance was observed between 'Zarjo' and 'Valfajr' cultivars that indicate their genetical kinship. The minimum genetic similarity observed between 'Sahand' and 'Makooee' populations indicates their genetical differences that might be due to mutation and/or selection. It is obvious that the measured genetic distance is a proportional distance and if the number of primers changes, the values will change too.

The relationship observed using molecular markers may provide information on the history and biology of cultivars, but it does not necessarily reflect what may be observed with respect to agronomic traits (Métais *et al.*, 2000). The selection process leads to an accumulation of best alleles for the traits under selection (Fernandez *et al.,* 2002). Further morphological and physiological investigations as well as genetic approaches will help to find the exact nature of these genetic variations.

Conclusions

In general, the results of this study showed that SSR markers have a high ability to reveal most of the information in a single locus and can be used for genetic analysis at molecular level determination of genetic similarity and clustering barley cultivars.

The amplification products were separated on 6% denaturing polyacrylamide gels containing 7M urea and visualized via silver staining method. High level of polymorphism was observed among populations. Polymorphic bands ranged from 100 to 300 bp. Altogether 65 alleles were observed among all genotypes, with an average of 9.2 alleles per locus for all loci. Polymorphic information content (PIC) ranged from 0.80 to 0.88 with an average of 0.84. 'Sahand' populations showed the lowest mean of gene diversity whereas the highest mean of heterozygosity observed in Rayhan populations that can prepare a powerful resource of genetic diversity for breeding programs. The genotypes were clustered using unweight pair-group

method on arithmetic average by POPGEN32 software. The dendrogram discriminated all the genotypes in several groups.

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