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Full Length Research Paper

The use of microsatellite markers for genetic diversity assessment of genus *Hordeum* L. in Kerman province (Iran)

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In this study, gene diversity and genetic relationships among 30 genotypes of genus *Hordeum* from Kerman province (Iran) were assessed using 10 simple sequence repeat (SSR) primers. Seven of these markers were highly polymorphic. A total of 96 alleles were detected. The number of alleles per microsatellite marker varied from 2 to 18. Except for Bmag0603, HVM36 and HvWAXY4, all the other markers showed on average, a high value of gene diversity ranging from 0.68 to 0.89. Primer GMS003 and GMS056 had the highest polymorphism information content (PIC) and so were able to distinguish different *Hordeum* genotypes. Cluster analysis based on unweighted pair group method with arithmetic averages (UPGMA) was accomplished by using the NTSYS-pc version 2.02 software for 4 major clusters which were finally extracted. Cluster 1 includes *Hordeum distichon, Hordeum vulgare* and *Hordeum spontaneum*, cluster 2 comprises *Hordeum leporinum* and *Hordeum glaucum*, cluster 3 has *Hordeum bulbosum* and cluster 4 includes *Hordeum brevisubulatum* ssp. *iranicum*. Data obtained from analysis are in complete agreement with taxonomic and gene pool classifications proposed previously. This study reports the first application of the SSR technique in characterization of genus *Hordeum* from Kerman province (Iran).

Key words: Microsatellite markers, *Hordeum*, gene diversity, genetic relationships.

INTRODUCTION

The genus *Hordeum*, belonging to the *Triticeae* tribe, has a wide distribution through the world and comprises 32 species and 45 taxa. Thirteen species are reported from Iran, of which 7 are found in Kerman province. It is one of the 4th world important crops and has been subjected to considerable genetics studies. Genus *Hordeum* is characterized by a spike with 3 single-flowered spikelets at each rachis node (Love, 1984). On the basis of morphological traits proposed by Von Bothmer and Jacobsen (1985), *Hordeum* has been divided into 4 sections, *Hordeum*, *Anisolepis*, *Stenostachys* and *Critesion* (Von Bothmer et al., 1991). Based on cross ability, the genus *Hordeum* can be divided into 3 genetic pools. The primary gene pool comprises *Hordeum vulgare*, *Hordeum spontaneum* and *Hordeum distichon*. The secondary gene pool is represented solely by *Hordeum bulbosum.* The tertiary gene pool comprises all the other *Hordeum* species. Cytological and molecular tools have been used extensively to study the relationships of the *Hordeum* species. Cytogenetic studies based on meiotic pairing among different diploid species of *Hordeum* showed four basic genomes: genome I common to *H. vulgare* and *H. bulbosum* (Kasha and Sadasivaiah, 1971; von Bothmer et al., 1983), genome X of the *Hordeum marinum* group (von Bothmer et al., 1986a, b), genome Y from *H.*

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Abbreviations: PCR, Polymerase chain reaction; **SSR,** simple sequence repeat; **GD,** genetic distances; **PIC,** polymorphism information content; **RFLP,** restriction fragment length polymorphism; **STS,** sequence-tagged site.

Table 1. List of *Hordeum* genotypes examined for genetic relatedness using SSR marker system.

No.	Code	Genotype	Location	No.	Code	Genotype	Location
1	H. vul1	*H. vulgare*	Joopar	16	H. gla3	*H. glaucum*	Kerman
2	H. vul2	*H. vulgare*	Dehbakri	17	H. gla4	*H. glaucum*	Estakhroie
3	H. vul3	*H. vulgare*	Jiroft	18	H. gla5	*H. glaucum*	Kohpayeh
4	H. vul4	*H. vulgare*	Mahan	19	H. gla6	*H. glaucum*	Baft
5	H. dis1	*H. distichon*	Baft	20	H. gla7	*H. glaucum*	Baghin
6	H. dis2	*H. distichon*	Baft	21	H. bre1	*H. brevisubulatum* ssp. *iranicum*	Baft
7	H. dis3	*H. distichon*	Kerman	22	H. bre2	*H. brevisubulatum* ssp. *iranicum*	Lalezar
8	H. spo	*H. spontaneum*	Sarasiab	23	H. bre3	*H. brevisubulatum* ssp. *iranicum*	Lalezar
9	H. spo	*H. spontaneum*	Estakhroie	24	H. bre3	*H. brevisubulatum* ssp. *iranicum*	Lalezar
10	H. spo	*H. spontaneum*	Sirjan	25	H. lep1	*H. leporinum*	Baghin
11	H. bul1	*H. bulbosum*	Dehbakri	26	H. lep2	*H. leporinum*	Estakhroie
12	H. bul2	*H. bulbosum*	Dokol	27	H. lep3	*H. leporinum*	Estakhroie
13	H. bul3	*H. bulbosum*	Maskoon	28	H. lep4	*H. leporinum*	Kerman
14	H. gla1	*H. glaucum*	Sarasiab	29	H. lep5	*H. leporinum*	Kabootarkahn
15	H. gla2	*H. glaucum*	Kerman	30	H. lep6	*H. leporinum*	Rafsanjan

murinum and the ge-nome H, shared by Asiatic, North and South American diploid barley (Dewey, 1984; Love, 1984; von Bothmer et al., 1986a). The genome designation was changed and Xa corresponds to X and Xu corresponds to Y according to von Bothmer et al. (1986a). Since the mid 1980s, ge-nome identification has progressed rapidly with the help of polymerase chain reaction (PCR) technology. Mole-cular polymorphism has been developed and used in genetic and breeding studies in many species of plants (Hu and Quiros, 1991; Svitashev et al. 1994; Bell and Ecker, 1994; Matsumoto and Fukui, 1996; Guilford et al., 1997; Haddad, 2008). Microsatellites as a marker have a high potential in using genome and genetic analysis of self-pollinating crops such as wheat, because of their high degree of polymorphism (Roder et al., 1995). Seve-ral studies on estimation of genetic diversity among *Hordeum* species have been carried out, for example, Graner et al. (1994), Saghai Maroof et al. (1994), Selbach and Cavalli-Molina (2000) and Struss and Plieske, (1998).

The objectives of this study are to define genetic diversity and relationship among species of the genus *Hordeum* using SSR markers and to determine the efficiency of the markers for further genome analysis.

MATERIALS AND METHODS

Plant material and DNA extraction

Leaf samples of 30 *Hordeum* genotypes (wild and cultivated forms) from Kerman (Table 1) were evaluated using 10 simple sequence repeat primers (Table 2). Genomic DNA was extracted by mini prep_ isolation method (Dellaporta and Hicks, 1983) with minor modifications. Approximately 0.2 g of young leaves from 8 mature plants of each accession was pooled for the extraction of genomic DNA. The leaves were frozen in liquid N_2, mixed with 400 µl of extraction buffer (50 mM Trisbase pH 8, 300 mM NaCl, 25 mM EDTA pH 8 and 1% SDS) and incubated at 65°C for 30 min. 200 µl sodium acetate 5 mM was added to each tube and placed about 10 min on ice. 500 µl chloroform/ isoamyl alcohol (24:1) was added and mixed well. The mix was centrifuged at 12000 g for 15 min. The supernatant was precipitated with an equal volume of ice-cold isopropanol and centrifuged at 5000 g for 15 min. At this stage, DNA was recovered by centrifuging. The pellet was hooked out by sterile pipettes, washed in 70% ethanol and air dried and suspend-ded in 300 µl of 1x TBE buffer. DNA quantity and quality were estimated by both using an UV spectrophotometer (Carry 50) by measuring absorbencies at A260 and A280 nm and 1% agarose gel electrophoresis by comparing band intensity with DNA ladder of known concentrations. DNA samples were diluted to 50 ng/µl for simple sequence repeat (SSR) reactions.

SSR reaction

PCR reactions were performed in 25 µl reaction volumes containing 2.5 µl PCR buffer (1x), 0.75 µl $MgCl_2$ (1.5 mM), 0.5 µl of each dNTPs (200 µM), 2 µl of each primer (10 pmol) , 0.2 µl of Taq polymerase (1 Unit) (Fermentas), 17.05 µl of deionize water and 2 µl of genomic DNA (50 ng/µl). Amplifications were performed in a gradiant thermal cycler (Eppendorf, Hamburg) and the program included 1 cycle of 4 min at 94°C, followed by 35 cycle of 1 min at 94°C, 30 s at 46 to 58°C (depending on primer) and 45 s at 72°C, followed by a final extention for 5 min at 72°C.

The PCR products were sepatated on sequencing gel containing 8% acrylamide (AA : BIS = 19:1) in 1x TBE buffer. For this 5 µl PCR product plus 2 µl loading dye (19 ml formamide (95%), 1 ml NaOH (10 mM), 0.05 g bromophenol blue (0.05%) (w/v) and 0.05 g xylene cyanol (0.05%) (w/v), were mixed and the gel was run for approximately 4 h at 100 W. After running, glass plate covering the gel was silver stained according to Bassam et al. (1991).

Data analysis

The amplified bands were scored manually as 0 (absent) or 1 (present). Genetic distances (GD) between samples were calculated using the DICE (equivalent to Nei and Li (1979) algorithm) based on the probability that the amplified fragment from one

Table 2. Primers sequences and annealing temperature of 10 *Hordeum* microsatellite markers.

Marker	Primer sequences (5´→3´)	Annealing temperature (°C)
GMS003	F: TTTCAGCATCACACGAAAGC R:TTGCATGCATGCATACCC	5
GMS027	F:CTTTTTCTTTGACGATGCACC R:TGAGTTTGTGAGAACTGGATGG	51
GMS056	F:GAGAAACGCAGCTGTGGC R:GTCACCGAGGCCTTCCTC	50
Bmag13	F:AAGGGGAATCAAAATGGGAG R:TCGAATAGGTCTCCGAAGAAA	60
Bmag0603	F:ATACCATGATACATCACATCG R: GGGGGTATGTACGACTAACTA	60
Bmac40	F:AGCCCGATCAGATTTACG R:TTCTCCCTTTGGTCCTTG	56
Bmac192	F:GGGTGGTGTTGCTTAAAC R:TCAACATTCATCTACCACCA	56
Bmac306	F:CCTTGTGTGAGTGTGTGTGT R:ACATGCACATGAACTAATCAA	59
HVM36	F:TCCAGCCGACAATTTCTTG R:AGTACTCCGACACCACGTCC	57
HvWAXY4	F:AGTATCGCAGACGCTCAC R:GTTATGTACTCGCTCGCTC	59

genotype will be present in another genotype, GD = 1-2a/2a+b+c, (a: number of shared fragments, b: number of fragments in line A, and c: number of fragments in line B). Dendrogram was constructed using Cluster method of the Unweighted Pair Group method with Arithmetic Averages (UPGMA) by NTSYS_ pc 2.02i (Rohlf 1998). Cut line was designed using molecular analysis of variance (MANOVA) method. Gene diversity (heterozygosity) was calculated according to Weir (1990):

Gene diversity = $1-\sum P^2ij$

Where, Pij is the frequency of the jth pattern for microsatellite marker and is summed across in patterns.

Anderson et al. (1993) suggest that gene diversity is the same as the polymorphism information content (PIC). Diversity can also be calculated by Shannon`s Information Index: $H' = -\sum P_i \ln p_i$, P_i is the allele frequency. These analyses were performed by POPGENE 32 software.

RESULTS AND DISCUSSION

The results of molecular assay in studying 30 *Hordeum* genotypes by use of 10 microsatellite markers are divided into 2 parts: 1) Determining the efficiency of markers and 2) genetic relationships among genotypes. All primers were also tested for their efficiency to reveal polymorphism in different *Hordeum* species by calculating allele length, number of alleles**,** PIC, heterozygosity and Shannon`s information index (Table 3). The amplified fragments length was from 102 to 379 bp. The number of alleles per marker varied from 2 to 18. PIC ranged from 0.29 to 0.89. Seven of the markers were highly polymorphic in this study. Except for HVM36, Bmag0603 and HvWAXY4, all other markers showed a high value of gene diversity (Table 3). GMS003 (Figure 1A) and GMS056 (Figure 1B) exhibited the highest gene diversity (0.89 and 0.80) among the markers, so these markers could separate the genotypes better and can be useful candidates for further studies. The number of alleles detected by microsatellite markers correlated with the gene diversity, for example the marker Bmag0603 showed an average gene diversity of 0.42, detecting 3 alleles within *Hordeum* genotypes, whereas GMS003 detected 18 alleles and showed a gene diversity value of 0.89. This finding was in contrast with Struss and Plieske, (1998) who used 15 microsatellite markers to estimate the genetic diversity among 163 barley genotypes. Hadad et al. (2008) working with grape also found correlation between number of alleles and gene diversity. Graner et al. (1990) and Selbach and Cavalli-Molina (2000) using restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) marker, respectively, observed a relatively low diversity within barley germplasm.

Genetic relationships among the accessions were further studied by cluster analysis (Figure 2). The dendrogram discriminated between 30 barley genotypes. The pattern of cluster analysis based on DICE similarity coefficient and UPGMA algorithm placed the genotypes into 4 major clusters. The cut line was at point 0.05.

Table 3. Allele length, number of alleles, gene diversity and Shannon`s information index of 10 microsatellite markers in 30 *Hordeum* genotypes.

Marker	Allele length in bp	Number of alleles	PIC (heterozygosity)	Shannon`s information index
GMS003	103-379	18	0.89	2.9201
GMS027	129-197	11	0.79	2.7013
GMS56	124-184	13	0.80	2.8468
Bmag13	150-186	7	0.68	2.0083
Bmag0603	127-188	3	0.42	1.2346
Bmac40	202-283	8	0.71	2.4592
Bmac192	133-187	10	0.73	2.8025
Bmac306	112-158	8	0.70	2.4602
HVM36	102-158	4	0.36	1.1129
HvWaxy4	127-183	2	0.29	1.0002

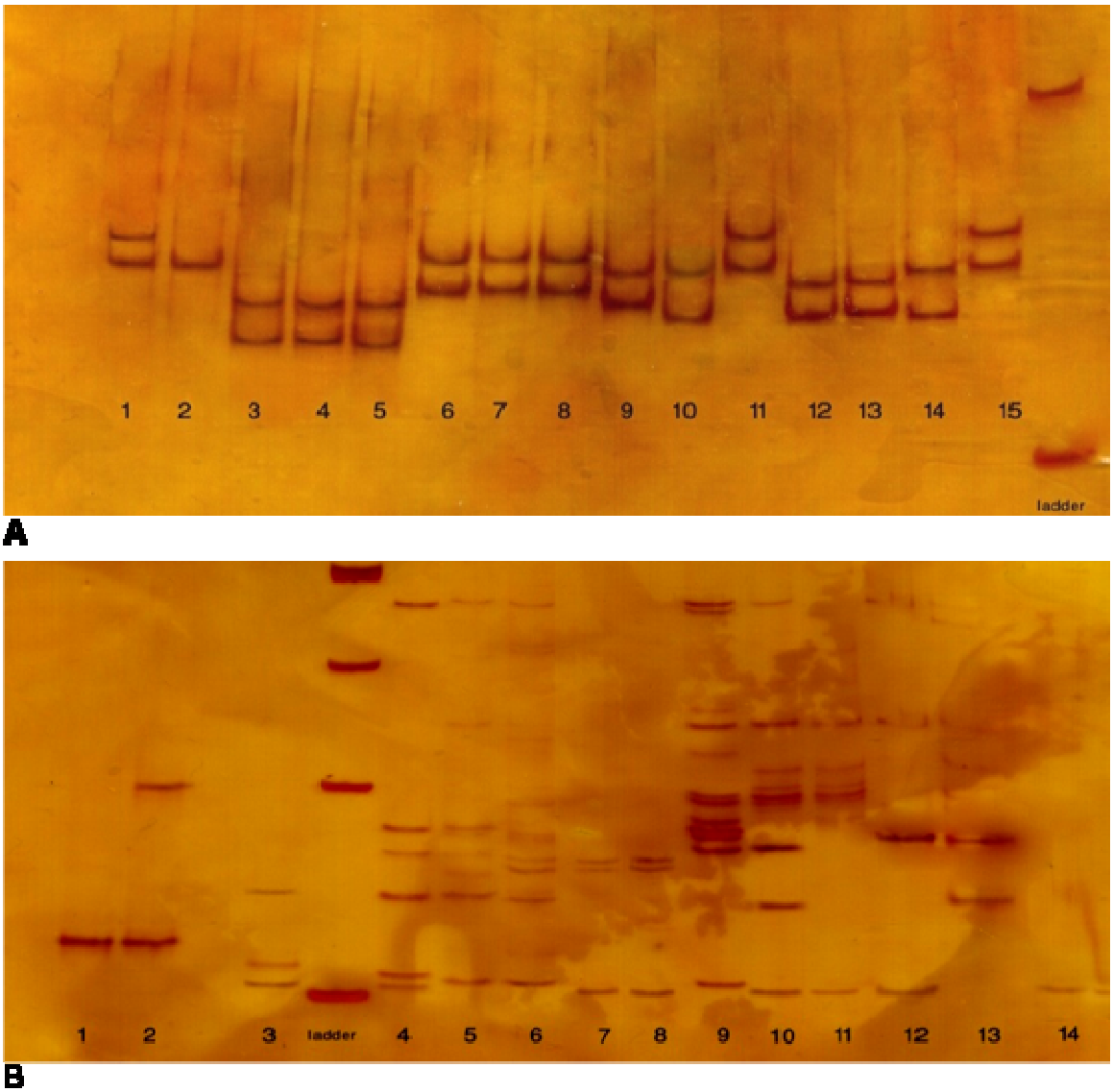

Figure 1. PCR product of microsatellite marker GMS056 showing 13 (A) and 18 (B) polymorphic alleles among 1 to 15 different *Hordeum* genotypes. The allele length is in bp

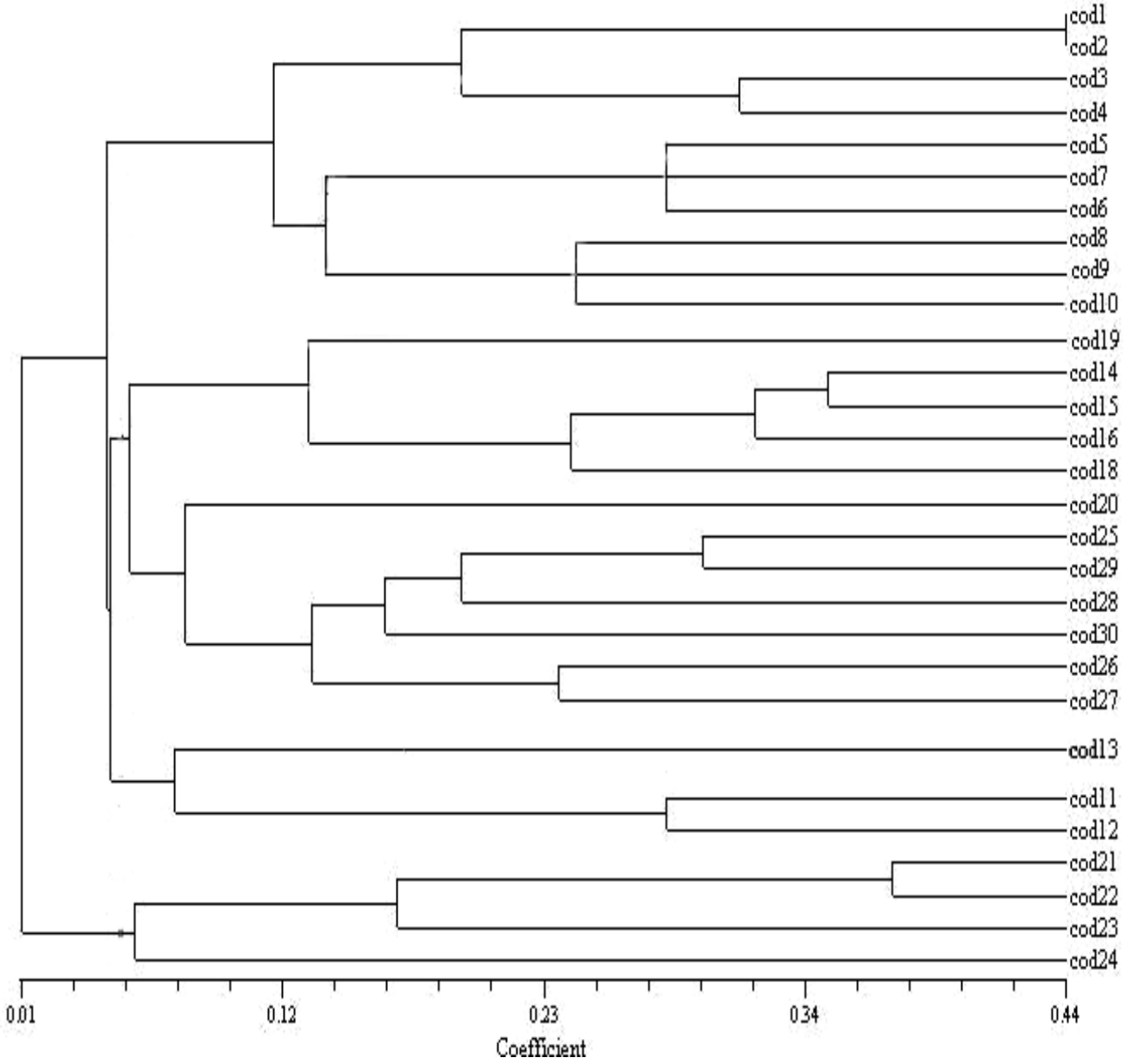

Figure 2. UPGMA dendrogram based on Dice similarity coefficient illustrating the relationships among 30 *Hordeum* genotypes.

Cluster 1 was represented by *H. distichon, H. vulgare* and *H. spontaneum*. Cluster 2 comprises *H. leporinum* and *H. glaucum*. Cluster 3 includes *H. bulbosum* as its sole representative. Cluster 4 comprises by *H. brevisubulatum* ssp. *iranicum.* Our result based on SSR markers was consistent with taxonomic classification based on numerous morphological (Bor, 1970), cytological and cross ability characters, delineated by and sequence-tagged site (STS) markers (Terzi et al., 2001). All *Hordeum* species with the I genome clustered together.

Our results grouped *Hordeum leporinum* and *Hordeum glaucum*, which all share the Xu genome according to von Bothmer et al. (1986a). Position of *H. bulbosum* confirms the findings of Svitashev et al. (1994), who used six cloned barely repetitive DNA sequences, regarding the clear distinction between *H. vulgare* and *H. bulbosum*. This was despite the attribution of the I genome to both species. In the dendrogram, all *Hordeum brevisubulatum* ssp. *iranicum* genotypes were grouped together, which was according to cross ability and cytological studies. Cluster analysis could clearly iden-tify the genetic relationships of the *Hordeum* genotypes examined in each set and demonstrates the potential and ability of microsatellite markers for genome analysis in the genus *Hordeum*. Totally, Our results using SSR markers are

in agreement with taxonomical classification based on cross ability and morphological studies (Sahebi, 2002), and cytogenetic studies based on meiotic pairing except for the *H. bulbosum* and molecular studies of Svitashev et al. (1994) and Struss and Plieske, (1998). In this study, microsatellite markers were able to distinguish between different *Hordeum* genotypes. The high gene diversity value of markers (with an average of 0.75) makes them ideal markers for differentiation between *Hordeum* geno-types. Similarly, high values of gene diversity were repor-ted for other plant species (Saghai et al., 1994; Rongwen et al., 1995; Szewc-McFadden et al., 1996; Guilford et al., 1997; Hernandez et al., 2001; Najafi et al., 2006; Hadad et al., 2008). The high degree of polymorphisms in our microsatellite markers is not only because of the germplasm tested but is also based on the variable cha-racter of microsatellites. The use of microsatellite mar-kers employed in this study demonstrates the usefulness of these markers for identification of *Hordeum* genotypes. They provide an optimal system which can increase the efficiency of germplasm evaluation in the *Hordeum* germplasm collection of Kerman province (Iran).

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