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GENETIC DIVERSITY AND STRUCTURE ANALYSIS IN WILD AND LANDRACES OF BARLEY FROM JORDAN BY USING ISJ MARKERS

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Abstract

The present experiment was carried out to estimate genetic diversity and genetic structure in cultivated and wild barley populations collected from Jordan which is considered as primary gene pool of barley. In a total, 94 cultivated barley accessions composed of 4 populations and 52 wild barley accessions consisted of 3 populations were used for genetic analysis using 7 Intron Splice Junction (ISJ) markers. The genetic diversity index (*He)* of cultivated barley ranged between 0.049 and 0.060; whereas that of wild barley populations ranged between 0.084 and 0.146, suggesting that wild resources of barley harbored greater genetic diversity than its domesticated counterpart, reflecting that barley domestication occurred with genetic bottleneck. Analysis of molecular variance showed high genetic variations among rather than within populations, referring that high genetic differentiation of barley populations caused by genetic and geographical separation of the populations in the harsh growing conditions of Fertile Crescent. Principal coordinate, clustering and STRUCTURE analysis not only separated cultivated and wild barley, but also each single population, showing their genetic basis and original sample site. The obtained results also revealed that there is lesser genetic communication between cultivated and wild barley under natural environments. The current findings can better be exploited for collection and utilization of plant germplasms.

Key words: ISJ markers, Genetic diversity, Barley, Jordan.

Introduction

Barley (*Hordeum vulgare* L.) has been cultivated since earlier times of human civilization, which makes it one of the earliest domesticated crop (Salamini *et al.,* 2002). The barley crop is best known for its ability of adaptation to wide range of environments and thus can be cultivated in semi arid to arid climates. For barley breeding, it is very important to recognize new and novel sources of variations. This will need a good perceptive of the existing genetic diversity both in cultivated and wild barley especially in the centre of domestication and diversification (Bothmer *et al.,* 2003). In this conext, Jordan is believed to be one of the countries of barley domestication where wild and landraces barley is widely distributed. Barley landraces are considered as more genetically diverse than modern cultivars due to its local adaptation in various environments (Fischbeck, 2003). In general, barley landraces are described as being very heterogeneous breeding material (Leino & Hagenblad, 2010). Barley landraces consist of valuable genetic resources of the cultivated barley in countries like Syria (Ceccarelli *et al.,* 1987), Ethiopia (Muhe & Assefa, 2011), Iran (Brown & Munday, 1982), Oman (Al-Dakheel *et al.,* 2012), Sardinia (Papa *et al.,* 1998), Turkey (Brush, 1995) and Jordan (Baloch *et al.,* 2014). Similarly, wild barley (*Hordeum vulgare* L. ssp. *spontaneum* C. Koch), the progenitor of cultivated barley acquire a great source of diversity (Nevo *et al.,* 1986a). It grows in various environments ranging from high rainfall to dry areas (Volis *et al.,* 2002). It has also been considered as useful source of untapped genes for breeding purpose in respect to biotic and abiotic stresses.

Landraces and wild barley from West Asia and North Africa were collectively studied for genetic diversity analysis by Orabi *et al.* (2009) using simple sequence repeat markers and found high variability among wild and landraces populations and further they suggested that both are interesting genetic resources for conservation and exploitation. Likewise, Wei *et al.* (2005), carried out an investigation with amplified fragment length polymorphism markers in landraces and wild barley collected from Near East Fertile Crescent and China and put forward that cultivar barley is distinct specie from wild progenitor. Weining & Henry (1995) applied intron splice junction (ISJ) primers to explore molecular diversity in *H. spontaneum* collected from Israel, Iran, Turkey and Pakistan. Results showed that the genetic variability in wild barley was largely associated with geographical distributions**.** ISJ primers were also used by Zeng *et al.* (2010) to compare the genetic variability between two sets of wheat from Tibet and concluded that these primers produced high amount of polymorphisms in different groups of wheat landraces. Recently, Drikvand *et al.* (2012) reported that ISJ primers could distinct two and six-rowed and also hulled and hulless barley genotypes.

Certainly, the collection of germplasm is being carried out at various levels, yet there is no such corresponding utilization of those valuable genetic stocks according to their potentiality for crop advancement by plant breeders. In this context, the efforts have been taken in the current study to estimate genetic diversity in landraces and wild barley populations so as diverse accessions can be utilized in future breeding programs.

Materials and Methods

Plant materials: In a total, 146 different accessions comprising of seven populations were used in the current study. Out of seven populations, four were two-rowed barley landraces, such as, Mafraq Manisha (MMHv), Madaba Team (MDHv), Mafraq Balwaneh (MBHv) and Showbak Gair (SGHv), while three of those were wild barley, namely, Tafila Al-Bada (THs), Showbak Gair (SGHs) and Ma'an Basta (MHs) (Table 1). These all genetic materials were collected and supplied by Prof. Hartwig H. Geiger, Institute of Plant Breeding, Seed Science, and Populations Genetics, University of Hohenheim, Stuttgart, Germany.

DNA extraction: About 5 seeds of each accession were randomly selected and sown in a growth chamber at a relative humidity of 75% and 25/20°C day/night temperature with light intensity of 3,000 lx. After 14 days, a single leaf of each accession was collected and used for DNA isolation. Genomic DNA was extracted as suggested by Weining & Henry (1995). Leaves were ground to a powder in 2mL microcentrifuge tubes under liquid nitrogen.Then, 600µl of sarkosyl buffer (containing 2% sodium lauroyl sarcosine, 0.1 M Tris-HCl pH 8.0, and 10 mM EDTA) was added and mixed well. The samples were incubated in ice-bath for 15 min after which 600µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the samples. Following centrifugation at 10,000 rpm for 10 min at 4°C, the upper phase was transferred to fresh tubes. Then 600µl chloroform was added and mixed. The samples were centrifuged for 10 min at 10,000 rpm at 4°C. The upper phase was transferred to fresh tubes, and then 500µl of isoproponol and 50µl 3M NaAc were added. The tubes were mixed and centrifuged (10,000 rpm, 1 min, and 4°C). The DNA pellet was washed twice with 70% ethanol, air dried and finally dissolved in 50µl of sterile ddH_2O. The DNA was quantified using a Nanodrop1000 spectrometer (Thermo Scientific, USA), and adjusted to a final concentration of 100 ng μL^{-1} with ddH_20 for PCR analysis.

Polymerase chain reaction and gel electrophoresis: Seven ISJ primers were used in this study (Table 2). PCR amplification was done in a total volume of 20 µl reaction mixture having 2.0 µl of 10 × buffer, 1.6 µl dNTP mix (2.5 mM), 1.6 µl $MgCl_2$ (25 mM), 2.0 µl primer (10µM), 0.4 µl of Taq polymerase (5 U/µl), 1.0 µl of template DNA (100 ng) and 11.4 µl of double-distilled H_2O. The amplification reaction of cycling program was performed as: initial denaturation for 2 min at 94°C, followed by 5 thermal cycles of 1 min at 94°C, 108 s at 40°C, 2 min at 72°C, followed by 29 cycles at 94°C for 1min, 58°C for 90 s, 72°C for 2 min. Final extension was done at 72°C for 10 min. The PCR amplified products were separated by electrophoresis in 3% agarose gels. The gel was run in 1×TAE buffer with voltage 120 for three hours. Later on, gels were put in ethidium bromide about 20 min for staining and then visualized and photographed under imaging system Gel Doc 2000TM (Bio-Rad) (Fig. 1).

Statistical analysis: Bands of ISJ markers were considered as 1 and 0 for the present and absent of bands, respectively, and the data produced was used for further statistical analysis. The GenA1Ex 6.5 software was used to work out genetic diversity of each population, while analysis of molecular variance (AMOVA) was also conducted through this software package in order to know the significant difference between barley populations. The significance of this F-statistic analogue was tested with 99 random per mutation. To investigate the population structure, the PCA analysis was first carried out with GenAlEx 6.5 (Peakall & Smouse, 2006) by three ways 1) all the accessions 2) barley landraces only 3) wild barley only. A genetic similarity matrix was calculated using DPS software (Tang *et al.,* 2012) by Jaccard methods (Jaccard, 1908) and the resulting neighbor- joining tree was displayed by MEGA v 5.10 (Tamura *et al.,* 2011). The model-based clustering program STRUCTURE v 2.3.4 (Pritchard *et al.,* 2000) was further inferred for population structure with a burn-in of 10000 iterations and run length with 10000 iterations. The delta K method (Evanno *et al.,* 2005) was employed to work out the optimal K (numbers of groups/clusters) for all the data set.

Table 1. Sample size and geographical data of barley populations collected from Jordan.

Population	Sample size	Latitude (decimals)	Longitude (decimals)	Altitude (meters)
MMHv	22	32.25	36.20	695
MDHv	24	31.68	35.83	785
MBHv	24	32.25	36.20	695
SGHv	24	30.30	35.30	1460
THs	14	30.80	35.62	1200
SGHs	17	30.30	35.30	1460
MHs	21	30.55	35.55	1420

Geographical data is based on Ghani *et al.* (2004)

Table 2. The 7 ISJ primers used for amplification of barley DNA.

Primer name	Sequence	Total number of bands scored	Genetic diversity (*He*) in *Hv*	Genetic diversity (*He*) in *Hs*
R1	5'-TCGTGGCTGACTTACCTG-3'	27	0.073	0.108
R2	5'-TGCTGGTTTGCAGGT-3'	19	0.131	0.096
R4	5'-TCGTGGCTGACTTACCTG-3'	41	0.066	0.111
R5	5'-TCGTGGCTGACGTCCATT-3'	37	0.055	0.098
E2	5'-GGAATTCCACGTCCA-3'	34	0.061	0.165
E4	5'-GGAATTCCACCTGCA-3'	39	0.014	0.097
E4-1	5'-GAATTCCAGCCTGCA-3'	39	0.107	0.110

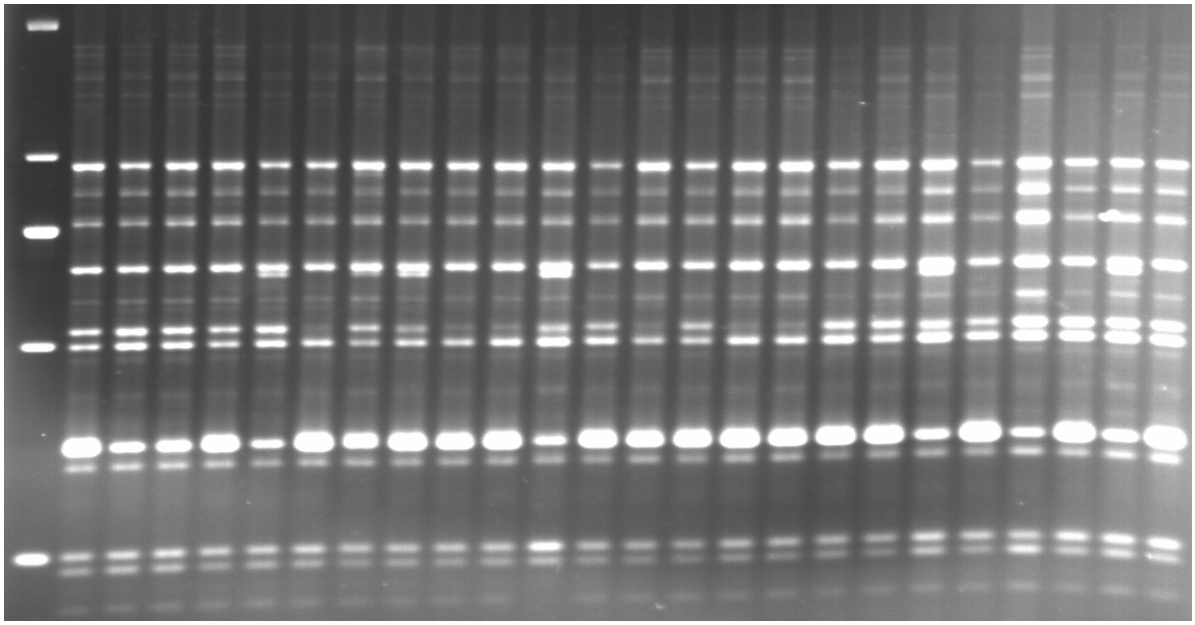

Fig. 1. DNA amplification patterns for 24 accessions of Showbak Gair landraces barley with E-2 ISJ primer.

Results

Distribution of alleles and marker diversity: Total seven ISJ markers previously documented by Weining and Langridge (1991) were used in the current study (Table 2). All ISJ primers showed polymorphism when considered over all populations and a total of 236 amplified fragments were detected. Primer R4 produced the largest number of alleles (41) and the lowest number of alleles (19) was shown by R2. Out of 236 alleles, 76 and 169 alleles were polymorphic in landraces and wild barley, respectively. As a whole, the distribution of genetic variability indicated that markers differ in their ability to manifest observed variability. The most diverse ISJ markers in landraces and wild barley were R2 (He = 0.131) and E2 (He = 0.165), respectively (Table 2).

Genetic diversity: A summary of molecular diversity based on ISJ markers is presented in Table 3. The estimation of the mean number of alleles per locus (*Na*), effective number of alleles (*Ne*), Shannon information index (*I*), the percentage of polymorphic loci (*P %*) and gene diversity (*He*) was done for each population separately. The mean values in landraces barley were: *Na* = 0.756 (range 0.716 – 0.788); *Ne* = 1.090 (range 1.100 – 1.081); I=0.083 (range 0.072-0.093); *He* = 0.054 (range 0.047 – 0.060); *P %* = 17.50 (range 15.25 – 19.92). The diversity parameters were largest in the MMHv population (*Na* = 0.788; *Ne* = 1.097; *I*=0.093; *He* = 0.060; *P %* = 19.92). Regarding wild barley populations, the average values were: *Na* = 0.997 (range 0.831 – 1.114); *Ne* = 1.195 (range 1.183 - 1.253); *I*=0.173 (range 0.124 - 0.217); *He* = 0.115 (range 0.146 – 0.084); *P %* = 33.20 (range 22.46 – 39.83). The highest genetic diversity was demonstrated by THs population (*Na* = 1.114; *Ne* = 1.253; *I*=0.217; *He* = 0.146; *P %* = 39.83). Intuitively, the values of *Na*, *Ne*, *I*, *He*, *P%* of wild barley were significantly higher than that of landraces. In landraces barley, the amount of genetic variability within and among populations showed that, on an average, 57% of the variation was within the populations and 43% was among the populations. With respects to wild barley, 46% of variation found within the populations whereas 54% of variation detected among the populations (Table 4). It is interesting that the main source of genetic variation is different between wild barley and cultivars.

Table 3. Within population genetic diversity estimates based on 7 ISJ markers in seven barley populations.

Population	Mean number of alleles per locus (N_a)	Effective number of alleles (N_e)	Shannon information index (I)	Genetic diversity (H_e)	Percentage of polymorphic loci
MMHv	0.788 ± 0.049	1.097 ± 0.015	0.093 ± 0.013	0.060 ± 0.009	19.92
MDHv	0.716 ± 0.047	1.081 ± 0.015	0.072 ± 0.012	0.047 ± 0.008	15.25
MBHv	0.742 ± 0.047	1.083 ± 0.015	0.076 ± 0.012	0.049 ± 0.008	16.53
SGHv	0.780 ± 0.048	1.100 ± 0.017	0.090 ± 0.013	0.059 ± 0.009	18.22
THs	1.114 ± 0.053	1.253 ± 0.024	0.217 ± 0.018	0.146 ± 0.013	39.83
SGHs	0.831 ± 0.050	1.148 ± 0.020	0.124 ± 0.016	0.084 ± 0.011	22.46
MHs	1.047 ± 0.054	1.183 ± 0.019	0.178 ± 0.016	0.115 ± 0.011	37.29

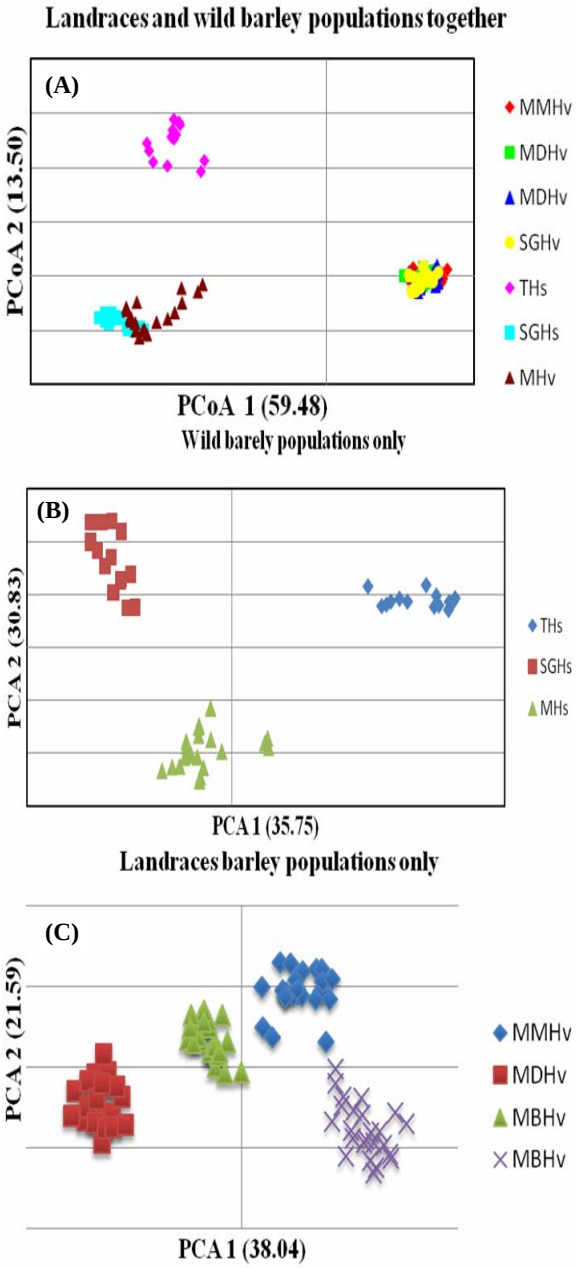

Fig. 2. (A) The PCA plot shows all the barley accessions of wild types and cultivars; (B) PCA plot shows wild populations only; (C) PCA plot shows landraces of barley only.

Principal coordinates analysis (PCoA): PCoA (Fig. 2A) analysis was performed to examine population structure throughout the entire accessions/individuals at the outset. Accessions of landraces mostly grouped together and samples from the MH and SG of wild populations were sorted into a relative tight cluster. Axis I, which explained 59.68% of the variation, separates landraces from wild barley. On axis II, THS was separated from the other two wild populations, which only account for 13.26% of the variation. The distribution with thinner span of landraces also implies high levels of genetic variation among wild accessions/individuals, which confirmed the former diversity analysis. However, in view of the fact that at the overall level PCoA can only distinguish wild from landraces but not single population/variety, therefore, we further separate them for deep analysis. In the PCoA plot for wild populations (Fig. 2B), three clear groups consistent with their predefined-populations were presented. The first and second principal coordinates accounted for 35.75% and 30.83% of the variation, respectively. Similarly, the landraces (Fig. 2C) were divided into four expected groups based on their original sample site in the PCoA plot for landraces only, with 38.04% for the axis I and 21.59% for axis II.

Genetic relationships: We sought to use the genome-wide genotypes across our collection of barley to elucidate the phylogenetic relationships between wild and landraces and to estimate the effects of micro-environment factors and geographic locations on barley diversity. The Neighbor-Joining (NJ) tree (Fig. 4) of all 146 individuals constructed through the UPMGA method, distinguished two subgroups, reflecting the wild populations and landraces. Furthermore, three clear sizable (>5 genotypes) taxa within the wild barley branch were detected, and under the branch of landraces, four taxa were distinguished, but two accessions (MBHv-22 and MBHv-23) from Mafraq Balwaneh and one accession (SGHv23) from Showbak Gair could not successfully cluster into their original population site as expected. When wild populations were compared with landraces, based on the NJ-tree, it is apparent that the genetic distance within landraces is far closer than that within wild, which also confirmed the high genetic diversity among wild barley. This phenomenon was supported by the Nei's Genetic Distance. The pairwise Nei's Genetic Distance (Table 5) with high identity was significant for all the comparisons between locations. Small differentiation was observed among landraces which ranged from 0.034 (MMHv-MBHv) to 0.073 (MDHv-SGHv), while for wild populations, the value ranged from 0.126 (MHs-SGs) to 0.214 (THs-SGs).

Structure analysis: We employed Bayesian clustering method as implemented in structure to detect the number of genetically similar groups or subpopulations (K) in the sampled isolated. The optimal number of clusters was detected by the delta K (ΔK) method (Fig. 3) and results showed that 2 was the best option from the broken line graph of ΔK. However, in order to detect the relationship between all the accessions and their pre-defined populations, we also explore the case that k=7. Under the fixed K (k=2, 7), one STRUCTURE run with the highest likeli-hood of the 20 runs was chosen to display the population structure. Results from figure 4 showed that all the accessions were assigned into two clusters; as a consequence, all landraces were grouped together and separated from wild barley. Low admixture between the two taxa showed the significant variation between them, which confirmed the preceding results. In addition to 7 clusters (Fig .4), all accessions were clustered into different groups that were consistent with their predefined populations representing their origins and sampling site. In contrast to k=2, several landraces depicted comparatively higher admixture. For example, the landraces developed from MMHv and MDHv, both showed a certain level of admixture with MBHv, this shows that hybridization and gene flow may happened during the long-term cultivation and acclimatization process among landraces, resulting in a certain degree of genetic correlation. Almost no mixture, however, was detected between wild barley populations.

Table 4. Analysis of molecular variance (AMOVA) of 146 accessions from seven barley populations based on 7 ISJ markers.

Source of variation	Degree of freedom	Variance components	Percentage of variation
Barley landraces			
Among populations	3	4.936	43
Within populations	90	6.648	57
Total	**93**	**11.584**	**100**
Wild barley			
Among populations	2	16.482	54
Within populations	49	14.199	46
Total	**51**	**30.681**	**100**
All populations			
Among populations	6	18.991	67
Within populations	139	9.310	33
Total	**145**	**28.300**	**100**
Mean among populations		13.469	54.66
Mean within populations		10.052	45.34

Table 5. Pair-wise population matrix of Nei's genetic distance among landraces and wild barley populations based on ISJ markers.

Population	MMHv	MDHv	MBHv	SGHv	THs	SGHs
MMHv						
MDHv	0.056					
MBHv	0.034	0.035				
SGHv	0.044	0.073	0.050			
THs	0.301	0.317	0.302	0.304		
SGHs	0.324	0.344	0.325	0.330	0.214	
MHs	0.259	0.269	0.264	0.256	0.175	0.126

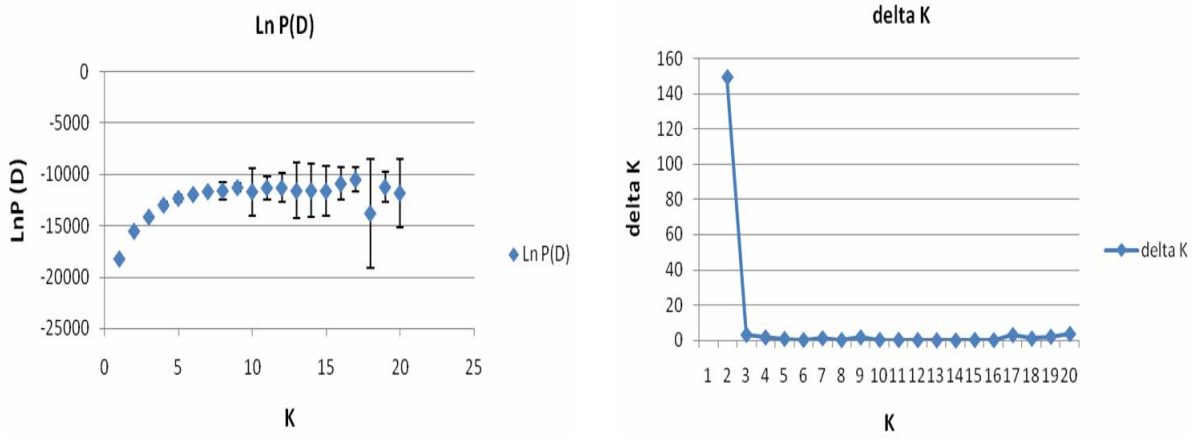

Fig. 3. The upper panels show the log likelihood profiles mean + SE Ln *P*(*D*) values based on 20 replicates with *K=1 to 20*, the lower panels show the rates of change in log likelihood for models with K=2 to 20, reffering the optimal K=2.

Discussion

For ongoing crop improvement attempts, it is very important to know the magnitude and distribution of genetic variability within barley germplasm. In the current study, the geographically matched samples were used to explore genetic diversity between landraces and wild barley and there were only fewer studies in which both species investigated together for diversity analysis. Our results based on genetic diversity parameters (Na, Ne, I, P%, He), genetic distance, AMOVA and PCoA demonstrate that higher diversity is present in *H. spontaneum* than in *H. vulgare*, indicating that a great attention is needed to preserve and exploit the genetic resources from wild barley. The results of current study were consistent with previous studies that wild barley harbor higher genetic diversity than cultivated barley. In this regard, Russel *et al.* (2011) assayed a large collection of genetically mapped, genome wide SNPs in geographically matched wild and landrace barley accessions from Fertile Crescent and reported that wild are more diverse than barley landraces. In contrast to our findings, Jana & Pietzrak (1988) carried out the other significant site matched comparison of landrace and wild barley within the Fertile Crescent. They observed that isozyme diversity in wild progenitors was somehow lower than in landraces materials across all 12 collection sites in Jordan. Matus & Hayes (2002) also

reported that SSR loci displayed a reduction of 50% diversity in cultivated than its wild progenitors. However, it is not surprising that wild progenitors of barley demonstrated high genetic diversity because it is believed that Jordan is one of the centers of domestication and diversification and this feature with other wild barley populations in this region has been demonstrated by various studies before (Weining & Henry, 1995; Baek *et al*., 2003; Shakhetrah *et al*., 2010). The loss of genetic diversity in landrace barley may be due to long term human mediated selection and the result of domestication since each domestication occurrence consequences in a genetic bottleneck and that results in narrowing the genetic base of crop plants (Tanksley & McCouch, 1997). Farmers are always keen to select the desirable traits in domesticated plants in comparison to their wild parents which cause drastic change in genetic constitution of cultivated crops. Conversely, wild progenitors have been grown under natural environmental where natural selection plays its key role, hence facilitating the selection for such characters that enhances fitness (Cleaveland & Solerki, 2007). We cannot, however, rule out the correlation of decreased population genetic diversity with decline in population fitness. Thus, reduction of genetic diversity in populations increases the chances of extinction. The low genetic diversity of barley landraces reflects that indigenous crop genetic reservoirs of Jordan are at the risk of extinction and may be lost before they are sufficiently collected, systematically evaluated and conserved.

Understanding the patterns of genetic differentiation among populations is very important for species protection and making effective conservation plans and it also helps in identifying units which need to be conserved (Allendorf & Luikart, 2007). The availability of variation among and within populations of crop species is necessary for their ability to survive successfully under dynamic changes of environments. As an average across all the populations, 54.66% of the total variation was observed among and 45.34% within the populations. The larger genetic variance among rather than within populations is likely due to self pollinated nature of the barley crop and the obtained results are in agreement with previous studies (Chalmers *et al.,* 1992; Ozkan *et al.,* 2005). According to Hamrick & Gadot (1989), reproductive system is the key factor in determining the genetic structure of plant populations. They reported that cross pollinated species tend to possess 10-20% of the total genetic variations among the populations; where as self pollinated crop species have about 50% of the total genetic variations among the populations. Contrary to above assumptions, other researchers found a greater amount of genetic variations within barely populations rather than among barley populations (Baum *et al.,* 1997; Turpeinen *et al.,* 2001, 2003). The higher genetic variations among populations imply that emphasis should be given to collect more populations so that genetic diversity can be maintained in this species. In addition, it would be better to conserve geographically different populations in order to control population declines caused by extensive environmental disasters. Furthermore, PCA was employed to analyze eco-geographical data, which has been used in previous studies on wild barley (Turpeinen *et al.,* 2001; Malysheva-Otto *et al.,* 2007). Also in our research, the results of PCA established that at the global scale, geographic origin elucidates the largest percentage of molecular diversity. Similarly, the UPGMA cluster analysis and the model-based clustering method when implemented in the structutre program also demonstrated a genetic structure pattern related to those predefined groups. Both the NJ tree and structure analysis showed that all the individuals can be clearly divided into two major clusters - wild and landrace. This finding was supported by pair-wise Nei's genetic distance, which suggested that 1) distinct variations exist between wild barley populations and cultivars; 2) compared to wild barley, landraces displayed a convergence of genetic diversity. These observations of group patterns could help to provide the possible structural description on barley. By this token, cultivars/landraces have been grown and selected by generations of farmers with strict restrictions during the domestication that resulted in a loss of genotypes and genetic variation, and sequentially, clustering analysis based on genetic differences between wild barley and landraces clearly divided them into two categories. However, the distinction within each subgroup was also observed. For landraces, genetic diversity between different varieties may be attributed to human selection according to their own preferences, such as malting, brewing, edible and so on. While the differences among the wild barley showed that the geographical location and micro-environment effect could be the imprinting factors to indicate the genetic structure. In the process of adaptation to the natural environment, wild barley not only retained a series of genes bearing on their growth, development and reproduction, but also generated a certain amount of new environment-related genes which resulted in the genetic differences among wild barley populations under the effect of different environments.

The existence of genetic contact between wild barley and cultivars under the natural conditions is still waiting for the inquiry. Happen to be in our experiment, there is a cultivar and wild population from the same region (SG), there is the possibility of gene flow during thousands of years of growth and cultivation process between them according to their geographical distance. However, our results suggest that is almost non-existent. For the following reasons 1) the genetic variation is significantly different. 2) PCA analysis could distinguish the two populations clearly. 3) They can be clustered into two different categories based on phylogenetic tree, and the genetic distance is different within each group, the average distance of wild population is loose, quit the opposite, the landraces showed a tightened genetic distance. 4) STRUCTURE analysis showed that when K=2, the two populations were assigned into two different cluster, and when K=7, they themselves formed a subgroup, and almost genetic similarity between each other. In summary, we believe that under natural conditions, there is little/no genetic communication between the wild and landraces barley that are grown in the same area. Seen in this light, it would be an important pathway that the genetic base of cultivars be broadened artificially using the wild genetic resources.

All in all, our study is useful for the collection, conservation and utilization of wild barley germplasm resources. Additionally it also provides a theoretical reference to broaden the hereditary base of cultivated barley.

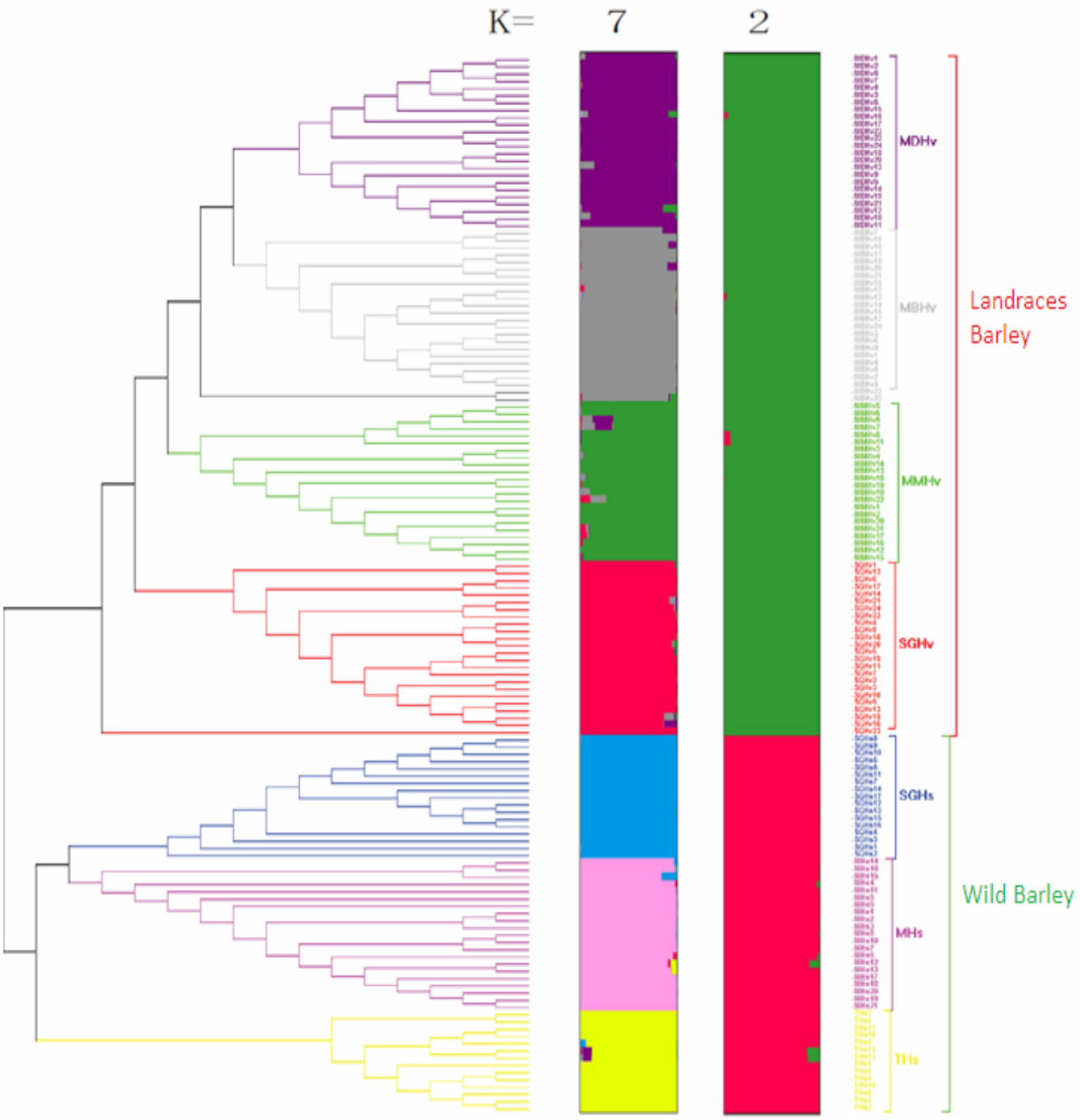

Fig. 4. NJ tree and population structure of landraces and wild barley as obtained from structure v 2.3.4 software.

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