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Discriminating between Barley (*H. vulgare* L.) Genotypes using Morphological and ISSR Markers

Adnan Kanbar

Department of Field Crops, Faculty of Agriculture, University of Damascus, Damascus, Syria.

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ABSTRACT

Genetic diversity assessment with different methods and their comparison could provide complementary information for improvement and conservation programs. In this study, a total of 9 morphological traits and 23 inter simple sequence repeat (ISSR) loci were used (i) to determine the effectiveness of discriminant analysis in recognizing Syrian cultivars from Japanese cultivars of barley on the basis of a few plant measurements, (ii) to classify the cultivars into groups based on molecular profiles and morphological traits. Analysis of discriminant analysis showed that number of leaves, leaf area index, root length and shoot dry weight were the most important variable for discriminating between the two barley groups. These four characters together were explained about 83.8% of the variability between two groups. By mean of these four predictors, 90% of the observations were correctly classified into their true groups as Syrian genotypes. Morphological and ISSR based clusters and their accompanying analyses showed different hierarchical patterns of genetic diversity among the genotypes. ISSR-based dendrogram in the present study revealed a powerful tool to quantify genetic diversity in barley, indicating very clear pattern of clustering according to the regions in which they are growing. The information on the genetic diversity relationship from this study is propitious to develop novel barley cultivars with desired economic traits.

Key words: Barley, discriminant analysis, genetic diversity, ISSR, morphological variation.

Introduction

Barley is considered one of the most important cereal crops not only in Japan and Syria but also all over world. It ranks as the fourth most important crop in the world after rice, wheat and maize. Barley is covering in Syria and Japan nearly 1,290,222 ha and 57,950 ha, produced 845,669 and 179,200 tones, respectively (F.A.O., 2010). It is very important feed supplement for domestic animals. The smallest a proportion serves directly for human nutrition in the form of barley (Zacharias, 2001).

Climate conditions play a main role in the evolution of landraces by demonstrating significant levels of variation in response to the selection stress in the regions (Souframanien and Gopalakrishna, 2004). Genetic architecture of a population is generally believed to be the result of breeding system, gene flow within and between populations, isolation mechanism and prolonged selection by various natural and artificial forces (Chandel and Joshi, 1983). The genetic variation within a taxon is not uniformly distributed throughout the geographic area where it is growing (Frankel *et al*., 1995) and populations from area far separated are normally expected to accumulate enormous genetic diversity (Chandel and Joshi 1983). Natural selection acting on heritable phenotypic variation will result in adaptation and differentiation among population of the same species inhabiting environments differing in their selective regime. In cultivated crop species, geographical distribution patterns reflect both the specific selection pressures prevailing in a particular environment as well as history of selection and production (Hawtin *et al*., 1997). Hence in diversity study, the inclusion of genotypes collected from different geographic areas has been adopted as a strategy to capture all sort of allelic diversity of a particular crop plant.

Determining the level of variation within, and among, barley populations is an essential step towards analysing genetic variability of cultivars (Singh 1996), select parental materials for hybridisation for making new genetic recombination, select inbred parents or tester for maximizing heterotic response and identify materials that should be maintained to preserve maximum genetic diversity in germplasm sources (Thormann and Osborn 1992). Several studies have been conducted to reveal the substantial level of genetic diversity within barley populations collected from farmers' fields (Kanbar and Kondo, 2011). Within the framework of these analyses, morphological markers (Lasa *et al*., 2001) as well as DNA markers (Tanyolac, 2003) have been

Corresponding Author: Adnan Kanbar, Department of Field Crops, Faculty of Agriculture, University of Damascus, Damascus, Syria.

employed. While morphological markers are inexpensive and easily implemented, DNA markers are not commonly affected by the environment and selection, and are also available in almost unlimited numbers (Ghebru *et al*., 2002). Among the various molecular marker techniques inter-simple sequence repeat polymorphic DNA (ISSR) has been widely used for genetic diversity studies in barley, maize and wheat (Tanyolac, 2003). In this study, the amount of genetic variation among and between Japanese and Syrian barley cultivars has been assessed using morphological and molecular marker tools.

Materials and Methods

Plant Materials:

A total of 20 barley cultivars, ten from each country (Syria and Japan) were used in this study (Table 1). All the ten Japanese genotypes were landraces, while there were two landraces namely; Arabi Abead and Arabi Asouad, in Syrian genotypes and the remaining were breeding varieties. All the genotypes are growing widely in both countries. They are having enormous morphological variability like grain shape, number of tillers, days to flowering, number of rows, maturity, resistance to abiotic and biotic stresses, etc.

Table 1: Names, character and origin of 20 cultivars of barley from Japan and Syria.

Syrian cultivars				Japanese cultivars			
Sr. No.	Names	character	Origin	Sr. No.	Names	characters	Origin
1	A. Asouad	Landraces	South Syria	1	Rensen Zairai	Landrace	Hokkaidou
2	A. Abead	Landraces	South Syria	2	Yuki Shirazu	Landrace	Miyagi
3	Furat 2	Breeder line	GCASR	3	Minamiuonuma Zairai	Landrace	Niigata
4	Furat 3	Breeder line	GCASR	4	Sekitori	Landrace	Toyama
5	Furat 4	Breeder line	GCASR	5	Yakko	Landrace	Saitama
6	Furat 5	Breeder line	GCASR	6	Miho	Landrace	Shizuoka
7	Furat 6	Breeder line	GCASR	7	Shirohada	Landrace	Kagoshima
8	Furat 7	Breeder line	GCASR	8	Bouzu	Landrace	Hiroshima
9	Furat 9	Breeder line	GCASR	9	Bouzu Mugi	Landrace	Tokushima
10	ACSAD 60	Breeder line	ACSAD**	10	Noumigun Zairai	Landrace	Ishikawa

*GCASR: General Commission for Scientific Agricultural Research.
**ACSAD: The Arab Center for the Studies of Arid Zones and Dry Lands.

Field Experiment:

Field experiment for the present study was conducted under the greenhouse conditions at the faculty of Agriculture, Damascus University, Syria, during summer 2010. Three barley seeds from each genotypes were directly sown in thick-plastic pipes measuring 50 cm in length and 20 cm diameter and opened from both sides. The pipes were filled with a mixture of sandy clay loam and FYM in 4:1 proportion. The soil was fertilized according to the recommended package of practices. The experiment was laid out in completely randomized design with three replications. The plants were watered daily to field capacity for 35 days after sowing after that sampling was done. Pipes were removed carefully and soaked in water for 4 hours to loosen the soil. After that, roots were cleaned thoroughly and carefully using a fine jet of water. The cleaned plant was collected in poly bags for recording observation. Observation consisted of Plant height (PHT) in cm, root length (RL) in cm, number of roots (NOR); number of leaves (NOL), total leaf area (LA) in cm^2, root fresh weight (RFW) in g and shoot fresh weight (SFW) in g. The plants were then oven-dried at 65^oC for 72 h and shoot dry weight (SDW) and root dry weight (RDW) were recorded.

DNA Extraction and PCR Amplification:

Thirty days old seedlings were used for DNA extraction. DNA extraction was done as per modified CTAB (Cetyltrimethylammoniumbromide) method (Cao and Oard 1997). The concentration and quality of DNA was estimated using spectrophotometer at 260 nm and 280 nm wavelength. A total of twenty-three ISSR were used in genetic analysis. The PCR reaction, the amplification profile and agarose gel preparation were described previously by Kanabr and Kondo (2011).

Statistical analysis:
General linear models:

The data of individuals was subjected to general linear model procedure to partite the variance using SAS statistical program (SAS Institute, Inc. 1996), and Duncan test was employed to classify mean values when F-values were significant ($P<0.05$).

Table 2: Polymorphism detected by 23 ISSR primers employed in the genetic diversity studies on two populations of Syrian and Japanese barley genotypes.

Primer	Sequence (5' 3')	Total bands	Polymorphic bands	Percentage polymorphism (%)
ISSR1	$(AC)_8G$	15	12	80.00
ISSR2	$(TC)_8C$	10	10	100.00
ISSR3	$(TG)_8G$	6	2	33.33
ISSR4	$(AC)_8GA$	11	10	90.91
ISSR5	$(GA)_8C$	15	15	100.00
ISSR6	$(AC)_8C$	14	9	64.29
ISSR7	$(GA)_8GG$	6	4	66.67
ISSR8	$(CA)_8AG$	6	5	83.33
ISSR9	$(CT)_8G$	8	7	87.50
ISSR10	$(GA)_8T$	7	5	71.43
ISSR11	$(AG)_8G$	8	4	50.00
ISSR12	$(CT)_8A$	5	1	20.00
ISSR13	$(TG)_8A$	5	5	100.00
ISSR14	$(AG)_8CTC$	9	6	66.67
ISSR15	$(AG)_8CTA$	12	6	50.00
ISSR16	$(GA)_8CTT$	9	5	55.56
ISSR17	$(GA)_8CTG$	6	4	66.67
ISSR18	$(CA)_8AGC$	5	4	80.00
ISSR19	$(AC)_8G$	12	7	58.33
ISSR20	$(AC)_8CTG$	8	6	75.00
ISSR21	$(ATG)_6$	9	8	88.89
ISSR22	$(GAA)_6$	4	1	25.00
ISSR23	$(AGT)_3(TC)7$	7	5	71.43

Step-wise Discriminant Analysis:

Step-wise discriminant analysis using PROC STEPDISC in SAS program (SAS institute, Inc., 1996) was employed to determine the best combination of variables that would separate between the two barley groups. The STEPDISC procedure selects a subset of quantitative variables to produce a good discrimination model using step-wise selection. The set of variables that make up each class is assumed to be multivariate normal with a common covariance matrix. Variables are chosen to enter or leave the model according to one of two criteria: (1) The significance level of an F test from an analysis of covariance, where the variables already chosen act as covariates and the variable under consideration is the dependent variable, or (2) the squared partial correlation for predicting the variable under consideration from the CLASS variable, controlling for the effects of the variables already selected for the model (Khattree and Naik, 1999).

Canonical Discriminant Analysis:

The data were also subjected to Canonical discriminant analysis using CANDISC procedure in the SAS program. Canonical discriminant analysis is a dimension-reduction technique related to principal component analysis and canonical correlation. PROC CANDISC derives canonical variables that summarize between-class variation in much the same way that principal components summarize total variation (Khattree and Naik, 1999).

Cluster Analysis:

Cluster analysis was performed based on morphological and DNA markers by using the STATISTICA software. The unweighted pair group method with arithmetic averages (UPGMA) and Squared Euclidean distances of the STATISTICA program were used to construct the matrices and the dendrograms using morphological and DNA markers.

Results and Discussion

A wide variability was observed for all root and shoot-related traits. Duncan test revealed significant differences between the two populations for root length, number of leaves, root fresh weight and root dry weight under study (Table 3). Mean performance of Syrian genotypes was higher than the Japanese genotypes for root length, number of root, number of leaves, root fresh weight, root dry weight and shoot dry weight. The evolution of landraces in distinct agro-climatic regions demonstrates significant levels of variation in response to the selection pressure in the regions (Singh *et al*., 1998; Souframanien and Gopalakrishna, 2004). Keeping in mind, in Japan, barley is planted in irrigated or high rain rate areas; while, in Syria, it's mostly planted rain-fed and in low rain fall areas and most of Syrian breeding lines are having good root system to be more tolerant to drought.

Table 3: Descriptive statistical analysis of morphological characters of two barley populations.

Variable	Group	Mean ± SE	Min.	Max.	Range	Mean square
Plant height	Japanese genotypes	22.56 ± 2.65 a	11.17	34.00	22.83	2.49^{ns}
	Syrian genotypes	21.85 ± 0.95 a	16.00	27.53	11.53	
Root length	Japanese genotypes	27.11 ± 1.45 a	20.67	33.67	13.00	728.06^{**}
	Syrian genotypes	39.18 ± 2.85 b	27.00	53.00	26.00	
Number of root	Japanese genotypes	7.35 ± 0.63 a	4.67	10.67	6.00	0.03^{ns}
	Syrian genotypes	7.43 ± 0.33 a	5.00	9.00	4.00	
Number of leaves	Japanese genotypes	4.00 ± 0.18 a	3.00	5.00	2.00	6.03^{**}
	Syrian genotypes	5.09 ± 0.15 b	4.33	5.67	1.34	
Leaf area	Japanese genotypes	22.38 ± 5.71 a	4.79	49.44	44.65	140.82^{ns}
	Syrian genotypes	17.08 ± 2.09 a	6.52	26.29	19.77	
Root fresh weight	Japanese genotypes	0.14 ± 0.02 a	0.04	0.27	0.23	0.04^{*}
	Syrian genotypes	0.24 ± 0.03 b	0.14	0.42	0.28	
Root dry weight	Japanese genotypes	0.02 ± 0.00 a	0.01	0.04	0.03	0.002^{**}
	Syrian genotypes	0.04 ± 0.00 b	0.03	0.07	0.04	
Shoot fresh weight	Japanese genotypes	0.52 ± 0.12 a	0.16	1.19	1.03	0.02^{ns}
	Syrian genotypes	0.44 ± 0.05 a	0.20	0.68	0.48	
Shoot dry weight	Japanese genotypes	0.07 ± 0.01 a	0.02	0.14	0.12	0.003^{ns}
	Syrian genotypes	0.09 ± 0.00 a	0.06	0.14	0.08	

Discriminant analysis result is presented in table 4. The most important variable for discriminating between the two barley populations was number of leaves with the partial R^2 52.9% followed by leaf area index 22.3% and root length 8.3%. These three characters together were explained about 83.8% of the variability between two groups.

Table 4: Step-wise discriminant analysis of root and shoot morphological traits between two populations of barley.

Step	Number in Character	Partial R-square	F value	Pr > F	Wilk Lambda	Pr < Lambda	Average squared canonical correlation	Pr > ASCC
1	Number of leaves	0.529	20.28	0.000	0.470	0.0003	0.529	0.0003
2	Leaf area	0.223	15.33	0.001	0.247	0.0001	0.752	0.0001
3	Root length	0.086	8.53	0.011	0.161	0.0001	0.838	0.0001
4	Shoot dry weight	0.048	6.21	0.021	0.113	0.0001	0.886	0.0001

A set of linear discriminant function were obtained from standardized variables (predictor variables were standardized to a mean of 0 and a standard deviation of 1) using number of leaves, leaf area index, root length and shoot dry weight as predictors (Table 5) and a corresponding classification table for this set was developed (Table 6). Variables were standardized so that the relative contribution of each component variable to the total compound discriminant score is indicated by the absolute magnitude of its corresponding discriminant coefficient. The discriminant coefficient for shoot dry weight was so high (Japanese group=131.79; Syrian group=310.72) comparing with the magnitude relative to root length (Japanese group=1.05; Syrian group=1.68). It is worth mentioning here that number of leaves manifested significant association with root length (data not shown), which is also reported to be one of the traits contributing towards drought tolerance. However, the root characters like root length cannot easily be monitored at field level. Hence, the characters such as number of leaves and shoot dry weight need to be given due emphasis while discriminating between diverse barley accessions from different locations.

Table 5: Coefficients for standardized linear discriminant functions using number of leaves, leaf area index, maximum root length and shoot dry weight as predictors of genotype populations.

Variable	Discriminant coefficients for group	
	Japanese genotypes	Syrian genotypes
Constant	-61.850	-127.938
Number of leaves	27.300	37.601
Leaf area	-1.080	-1.890
Root length	1.050	1.680
Shoot dry weight	131.790	310.720

By mean of number of leaves, leaf area index, root length and shoot dry weight as predictors, 95% of the genotypes were correctly classified into their true groups (Table 6). However, a low error rate was observed in the classification of Syrian genotypes (10%). The separation of Syrian genotypes from Japanese genotypes was not improved significantly by adding the remaining measured characters to the predictors. Over all correct classification by all the characters as predictors has reduced insignificantly from 95% to 90% (5% differences) (Table 7). Thus, the contribution of four traits to classify the genotypes into their true groups was equal to the contribution of all others. Ebdon *et al*. (1998) used discriminant analysis in identification of low and high water use Kentucky bluegrass cultivars. They reported that a fewer measurement or predictors *viz.*, leaf angle and leaf

area, more efficient to perform classification into their true water use groups. Discriminant analysis was also used to select deep-rooted F_2 lines based on a few shoot-related traits in rice (Kanbar *et al*., 2010).

Table 6: Summary of classification with cross-validation using number of leaves, leaf area index, root length and shoot dry weight as predictors of barley genotype populations.

	True group		Predicted group membership	
Group	Number of cases		Japanese genotypes	Syrian genotypes
Japanese genotypes	10		10	0
Syrian genotypes	10		1	9
Proportion correct (%)			100%	90%
Total correct (%)			95.00	
Error rate			0.00	0.10
Total error			0.05	

Table 7: Summary of classification with cross-validation using all nine characters as predictors of barley genotype populations.

	True group		Predicted group membership	
Group	Number of cases		Japanese genotypes	Syrian genotypes
Japanese genotypes	10		10	0
Syrian genotypes	10		2	8
Proportion correct (%)			100%	80%
Total correct (%)			90.00	
Error rate			0.00	0.20
Total error			0.10	

Different methods of genetic diversity measures could give better judgment of differentiating important accessions for growers, germplasm curators and plant breeders. Data of 20 genotypes of barley were used to assess their genetic diversity, employing morphological and inter simple sequence repeat (ISSR) marker methods. The analysis of genetic diversity through the cluster analysis based on morphological characters has been shown in Fig. 1a.

Cluster diagram based on unweighted pair group method with arithmetic averages (UPGMA) and Squared Euclidean distances of the STATISTICA program were used to construct the matrices and the dendrograms (StatSoft, Inc., 2003). The genotypes were categorized into four clusters at 15% linkage distance, cluster I consisted two genotypes, cluster II nine, cluster III five and cluster IV four genotypes. All the four clusters were analyzed for mean and SD (Table 8). Distribution pattern of all the genotypes into various clusters showed the presence of considerable genetic divergence among the genotypes for most of the traits studied. Maximum and minimum genetic distances were observed within cluster IV and II, respectively. Variability within these cultivars was largely influenced by the breeding system and climatic conditions. This analysis shows that there is partial relationship between the geographic origin and their divergence on the basis of all morphological traits studied. Genetic divergence among barley genotypes through cluster analysis was also reported by Bahrman *et al*., 1999; Hamza *et al*., 2004; Eshghi and Akhundova, 2010.

Table 8: Mean and standard deviation of four clusters for nine characters.

Characters	Mean ± SD			
	Cluster I	Cluster II	Cluster III	Cluster IV
Plant height	25.26 ± 3.20	17.16 ± 0.10	22.26 ± 1.40	31.94 ± 2.01
Root length	27.33 ± 0.47	28.63 ± 6.38	46.13 ± 6.12	30.00 ± 3.60
Number of root	8.00 ± 1.41	6.15 ± 1.08	7.73 ± 0.49	9.46 ± 0.87
Number of leaves	5.50 ± 0.24	4.03 ± 0.69	5.13 ± 0.50	4.50 ± 0.43
Leaf area	22.42 ± 5.47	8.87 ± 2.84	19.82 ± 3.91	42.70 ± 6.80
Root fresh weight	0.27 ± 0.01	0.11 ± 0.05	0.27 ± 0.12	0.24 ± 0.020
Root dry weight	0.03 ± 0.00	0.02 ± 0.01	0.05 ±0.01	0.02 ± 0.00
Shoot fresh weight	0.66 ± 0.02	0.25 ± 0.05	0.46 ± 0.08	0.94 ± 0.17
Shoot dry weight	0.12 ± 0.02	0.05 ± 0.02	0.09 ± 0.02	0.11 ± 0.02

Since morphological markers are prone to equivocal interpretations and time consuming, not always available for analysis and are affected by changing environmental conditions, molecular marker technology offers several advantages over the sole use of conventional markers in cultivar identification and breeding programs. Molecular markers help to distinguish labeling mistakes, identification of the genuine owner of the cultivar in question and routine identification of cultivars in nurseries. Twenty-three ISSR primers were used to amplify the DNA of all the 20 barley genotypes and totally yielded 197 bands which contained 141 polymorphic bands (Figure 1b). The result of ISSR amplification products and polymorphism level was shown in Table 2. Length of the ISSR amplification products ranged from 300 bp to 2,400 bp. The number of polymorphic bands generated by each primer ranged from 1 to 15 with an average of 5.9. The primer ISSR5 with sequence of $(GA)_8C$ and the primer ISSR1 with sequence of $(AC)_8G$ detected 15 and 12 polymorphic loci among the 20 cultivars, respectively. It indicated that all 20 barley cultivars could be distinguished by ISSR primers. The

resulting dendrogram clustered into two major clusters; one for the Syrian cultivars and the other for the Japanese cultivars. However, two sub-clusters formed part in the two main clusters. Dendrogram in the present study indicated very clear pattern of clustering according to the regions in which they are growing. Similar results were obtained in barley (Russell *et al*., 1997; Fernández *et al*., 2002; Bahattin, 2003). The genetic closeness among the cultivars of each group can be explained by the high degree of commonness. The higher levels of genetic variation found in this study may be due to the very diverse geographic structure in Japan and Syria and the high degree of climatic heterogeneity of Syria (semi-dried area) compared to Japan (Sub-tropical climate). Geographically isolated population accumulates genetic differences as they adapt to different environment (Souframanien and Gopalakrishna, *et al*., 2004).

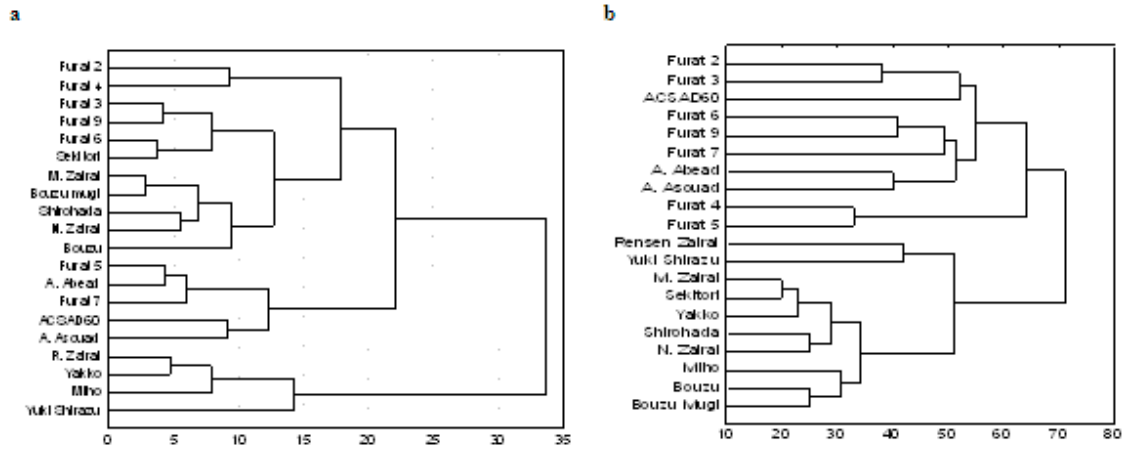

Fig. 2: Dendrogram showing grouping of 20 genotypes from two populations of barley based on (B) the genetic distance derived from ISSR markers and (A) morphological characters, using UPGMA analysis.

In conclusion, morphological and ISSR based clusters and their accompanying analyses showed different hierarchical patterns of genetic diversity among the accessions. Despite their disparity, the two diversity measures were found independently useful for assessing the degree of relatedness and the overall patterns of genetic variation among the analysed barley cultivars. However, the ISSR markers were found to be more effective in grouping barley genotypes. Furthermore, the wider phenotypic and molecular variability observed represents a good indication for the importance of barley genotypes in breeding programs.

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