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Genetic Characterization of Barley Genotypes via Inter-Simple Sequence Repeat

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***Abstract*—**In this study, polymerase chain reaction based Inter-simple sequence repeat (ISSR) from DNA fingerprinting techniques were used to investigate the genetic relationships among barley crossbreed genotypes in Turkey. It is important that selection based on the genetic base in breeding programs via ISSR, in terms of breeding time. 14 ISSR primers generated a total of 97 bands, of which 81 (83.35%) were polymorphic. The highest total resolution power (RP) value was obtained from the F2 (0.53) and M16 (0.51) primers. According to the ISSR result, the genetic similarity index changed between 0.64–095; Lane 3 with Line 6 genotypes were the closest, while Line 36 were the most distant ones. The ISSR markers were found to be promising for assessing genetic diversity in barley crossbreed genotypes.

***Keywords*—** Barley, crossbreed, genetic similarity, ISSR.

I. Introduction

CERAL production in the world and in Turkey has a great importance and weight than other agricultural products in economic and social life. Feed grains in the number of animals in need of barley grain and hay production and at the same time meet has an important role in the production of malt. Therefore, in order to constant and steady increase in production, the use of suitable varieties and cultivation techniques in addition to varieties that can be grown in different climates and soil conditions is important. The achievement of a breeding study is determined by the genetic variability available in the germplasm pool of the crop [3].

Molecular markers commonly use plant breeding and molecular genetic research in many laboratories worldwide [5]. Many studies have indicated that DNA-based markers (especially ISSR, RAPD, and SSR) are useful in describing the genetic relationships among closely related barley genotypes and its cultivars.

In research of called “Inter-simple sequence repeat (ISSR) markers and some physiological attributes of barley (*Hordeum vulgare* L.) genotypes to drought and potassium nutrition” was designed to identify useful effects of potassium for drought tolerance in barley [1]. Wang *et al.* [15] were investigated genetic diversity in wild close relatives of barley from Tibet and the Middle East by ISSR and SSR markers.

Barley cultivar discrimination and hybrid purity control were carried out by Mylonas *et al.* [8] using RAPD markers. It was stated that four RAPD markers selected from ten different oligonucleotides could be used to facilitate breeding programs.

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Karim *et al.* [7] were analyzed genetic diversity and relationships in local Tunisian barley by RAPD and SSR analyses. Similarly, genetic identification was performed by Hayden *et al.* [5] in barley (*Hordeum vulgare* L.) germplasm using informative DNA markers.

El-Awady *et al.* [17] used ISSR and RAPD markers for genetic variability determination of Saudi barley genotypes. Both the marker systems were robust to identify the studied genotypes. Nonetheless, the ISSR results were more informative compared to RAPD results regarding the geographical distribution of the six barley landraces. Therefore, the outcome of this investigation can help to strengthen the current germplasm data on barley that may help identify national barley programs.

Sofalian and Behi [18] have studied the freezing resistance and genetic diversity in 20 barley genotypes using molecular markers. Researchers stated the analysis of genetic variability showed a significant difference among genotypes and ISSR could be used to distinction or classification of barley genotypes as an effective marker system in breeding.

Tanyolaç [13] investigated the variation among wild barley (*Hordeum spontaneum* Koch) populations from west Turkey by ISSR and RAPD, the two dominant markers. RAPD and ISSRs were effective and promising marker systems for detecting genetic variation. The results of the mentioned study demonstrated that genetic variation existed within broomrape (*Orobanche*) species according to RAPD results.

Fernandez *et al.* [2] investigated the genetic diversity among barley cultivars with known origin using of ISSR and RAPD markers. Bulk analyses of RAPD and ISSR markers provided a rapid, credible and highly informative system for DNA fingerprinting and showing clusters that separate very well the spring/winter and six rows/two-rows cultivars. The dendrograms obtained with these markers were conformity with their known origin.

In this study, hybrid barley genotypes obtained from a breeding program was characterized by molecular markers and the genetic relationship between the hybrid genotypes and their parents.

II. Materials and Methods

A total of 39 genotypes were used in the study, including four barley genotypes, two of which are proprietary (*Hordeum vulgare* L. cv. Bülbül 89 and Karatay 94) and two local varieties (Hulles barley-*Vibirnum nudum* L. and Wild barley-*Hordeum spontaneum* Koch.), and 35 lines selected from "full diallel" advanced hybrid lines. Some characteristics of these genotypes are given in Table I.

TABLE I
SOME CHARACTERISTICS OF BARLEY GENOTYPES

Genotypes	Lane No	Seed status	Glume Color	Grain Color	Plant length (cm)	Spike duration (day)
Bülbül 89	28	Hulled	White		100	120
Karatay 94	30	Hulled	White		100	120
Hulless barley (*V. nudum* L)	38	Hulless		Colored Aleuron	85	103
Wild barley (*H. spontaneum* Koch.)	7	Hulled	Black		90	108
Bülbül 89 x Hulless	15	Hulless		Yellow	96	115
	12	Hulless		Yellow	94	106
	39	Hulless		Grey	91	102
	23	Hulless		Yellow	94	110
Hulless x Bülbül 89	1	Hulled	Brown		99	108
	3	Hulless		Yellow	94	106
	8	Hulless		Brown	100	106
	6	Hulless		Brown	95	103
	4	Hulless		Yellow	96	104
	5	Hulless		Brown	101	104
	27	Hulless		Yellow	87	103
	16	Hulless		Colored Aleuron	90	109
	17	Hulless		Brown	94	104
	24	Hulless		Brown	92	103
	31	Hulless		Brown	78	101
Bülbül 89 x Wild	10	Hulled	Black		85	103
	13	Hulled	White		86	100
	36	Hulled	Black		86	100
Karatay 94 x Hulless	14	Hulless		Brown	86	115
	19	Hulless		Yellow	101	115
	20	Hulless		Brown	93	115
	22	Hulless		Brown	100	106
	32	Hulless		Yellow	110	120
	29	Hulless		Yellow	100	105
	18	Hulless		Brown	105	120
	11	Hulled	White		94	107
	2	Hulless		Brown	93	106
	34	Hulless		Brown	95	102
Hulless x Karatay94	26	Hulled	Brown		105	120
	33	Hulless		Brown	111	120
Karatay 94 x Wild	35	Hulled	Black		103	103
	25	Hulled	Black		97	105
	21	Hulled	Brown		109	120
Wild x Hulless	9	Hulless		Yellow	83	100
	37	Hulless		Yellow	75	104

A. DNA Isolation

Seedlings were grown in a growth chamber. For each genotype, leaves of 10 plants (3 wk old) were mixed to create a bulk from which 200 mg were used for DNA isolation. Genomic DNA was extracted using a modified 2x CTAB method. DNA concentrations were determined by a Nanodrop BioPhotometer. DNA samples were run on 1% (w/v) agarose (Prona) gels. Out of 20, 14 ISSR the most suitable primers (in terms of repeatability, scalability and the ability to distinguish between accessions) were selected for identification (as shown in Table II).

B. ISSR Assay

Each ISSR reaction mix contained 2.5 mM $MgCl_2$ (Fermentas), 1x PCR Buffer (Fermentas), 2.5 µM of each of dNTPs (Fermentas); 0.5 µM primer, 2 µL of 25 ng DNA template and 0.3 units of Taq DNA Polymerase (Fermentas) in a final reaction volume of 25 µL was prepared. The PCR reaction was performed in a touchdown fashion with a first denaturation at 94°C for 5 min, followed by; (1) denaturation at 94°C for 1 min, (2) annealing at temperature melting (Tm) for 1 min, and (3) extension at 72°C for 2 min, with the annealing temperature being diminished by 0.5°C per cycle. This procedure was followed by; (1) denaturation at 94°C for 1 min, (2) annealing at Tm for 1 min, and (3) extension at 72°C for 2 min, and a final extension at 72°C for 10 min. All PCR reactions were completed 40 cycles and repeated at least twice. 15 primers producing consistent and polymorphic fragments were selected for PCR amplification. Upon completion of the reaction, amplified products were electrophoresed on a 2% (w/v) agarose/1x Tris-Borate-EDTA gel and followed by visualization in a Vilber Lourmat (France) device.

TABLE II
CHARACTERISTICS OF OLIGONUCLEOTIDES USED IN DEGENERATE AND INVERSE PCR REACTIONS FOR ISSR ASSAYS

ISSR	Sequence (5'-3')	Tm (°C)	Primer length (bp)	G/C (%)
M1	$(AGC)_6G$	63.1	19	68.4
M5	$(GA)_9C$	56.7	19	52.6
M7	$(AG)_9C$	56.7	19	52.6
M8	$(AC)_9G$	56.7	18	52.6
M9	$(AC)_8CG$	56.0	18	55.6
M11	$(CAC)_5$	53.3	15	66.7
M15	$(CA)_8AG$	53.7	18	50
M16	$(CA)_8GC$	56.0	18	55.6
M17	$CAG(CA)_8$	56.7	19	52.6
M18	$CGT(CA)_8$	56.7	19	52.6
F1	$GAG(CAA)_5$	49.1	18	38.9
F2	$CTC(GT)_8$	56.7	19	52.6
F3	$(AG)_8CG$	56.0	18	55.6
F6	(CCA)5	53.3	15	66.7

Genetic similarity among the genotypes was estimated on the basis of the ISSR patterns resulted from the primers with the genomic DNA of representative genotypes, following the similarity coefficient of Jaccard [6], in which the polymorphism information content (PIC) of each marker was counted with a modified method of the original formula.

$$PIC = 1-\sum P_i^{\,2}$$

where P_i is the band frequency of the ith allele [12]. Marker Index (MI) was calculated as described by Grativol *et al.* [4].

$$MI=EMR \times PIC$$

EMR (effective multiplex ratio) is the product of a total number of fragments per primer (n) and the fraction of polymorphic fragments (β):

$$EMR = n \times \beta$$

The resolution power (RP) of primers was calculated from the formula:

$$RP = \sum I_b, \quad I_b = 1 - (2 \times |0.5 - p|)$$

where p is the rate of I band in all genotypes [9].

C. Data Analyses

ISSR assays were repeated at least twice for each primer, and only the reproducible fragments were scored, with special emphasis on the repeatability of the bands that present polymorphism. Each DNA fragment generated were treated as a separate character and scored accordingly (1 for the presence and 0 for the absence). A rectangular binary data matrix of 15 x 64 was prepared, and its statistical analysis was performed using the NTSYS-pc (Ver 2.2; [11]). In cluster analysis, the unweighted pair group method with the arithmetic mean (UPGMA) procedure was followed.

III. Result And Discussion

A. DNA Polymorphism and Marker performance

As a result of PCR analysis with ISSR primers, ninety-seven bands were obtained from fourteen primers in total, and eight of these bands became polymorphic. F2, M1, M2, M8, M9 and M16 primers showed one hundred percent polymorphism. (as shown in Table III).

TABLE III
PCR Amplification Result of ISSR Primers

Primer	Number of bands	Polymorphic bands	Polymorphism (%)	PIC	MI	RP
F1	6	4	66.7	0.825	3.30	0.192
F2	6	6	100.0	0.669	4.01	0.530
F3	5	4	80.0	0.490	1.96	0.397
F6	5	3	60.0	0.272	0.82	0.308
M1	16	16	100.0	0.281	4.50	0.173
M5	10	7	70.0	0.232	1.62	0.271
M7	14	13	92.9	0.224	2.91	0.197
M8	3	3	100.0	0.694	2.08	0.442
M9	11	11	100.0	0.425	4.68	0.378
M11	4	3	75.0	0.296	0.89	0.342
M15	4	2	50.0	0.394	0.79	0.513
M16	5	5	100.0	0.850	4.25	0.492
M17	4	2	50.0	0.320	0.64	0.385
M18	4	2	50.0	0.470	0.94	0.359
Total	**97**	**81**	**-**	**-**	**-**	**4.98**
Mean	**4.85**	**4.05**	**91.63**	**0.62**	2.39	**0.36**

The information on the genetic profile of each accession obtained using the fourteen ISSR primers was used to assess the marker performance through evaluation of three parameters: polymorphic information content (PIC), marker index (MI) and resolution power (RP).

To determine PIC values of each ISSR primer, we analyzed the mean of PIC values for all loci of each ISSR primer. As a result, we obtained high values of PIC for the ISSR primers M16 (0.850) and F1 (0.825) and a low PIC value for the ISSR primer M7 (0.224). The average value of PIC per primer was 0.62.

Marker Index values (MI) of ISSR primers used in this study were observed/ calculated between 0.82 and 4.68. The highest MI value was obtained from the M17 primer (0.64), and the lowest MI value was calculated from the M9 primer. The total MI value of 14 primers for barley genotypes was 2.39 in the study. The marker index (MI) may be used to evaluate overall utility of a marker system [10]. In a study [14] were indicated that ISSR has higher MI in comparison to RAPD in triticale genotypes.

When the 14 ISSR primers were compared in terms of resolution powers, it was determined that the total RP power value of the primers was 4.98 and the mean RP value was 0.36. The lowest value for the total RP value was obtained from the M1 primer (0.173), and the highest total RP value was obtained from the F2 (0.53) and M15 (0.51) primers. It is known that the resolving power (RP) is a procedure employed to measure the capacity of primers or techniques to identify genotypes [9]; [14].

If we group the genetic similarities between the barley genotypes, genetic similarity coefficients are formed between eighteen genotypes 0.79-0.86, fourteen genotypes 0.71-0.78, three genotypes 0.64-0.70, and 2 genotypes 0.95.1.00 (as shown in Table IV).

TABLE IV
Grouping of Genetic Similarity Coefficients among Barley Genotypes

Genetic similarity coefficients	Between Genotypes	
	Number	%
0.95-1.00	2	5.13
0.87-0.94	-	-
0.79-0.86	18	46.15
0.71-0.78	16	41.03
0.64-0.70	3	7.69
Total	39	100

B. UPGMA Dendrogram of among the Barley Genotypes

As a result of the analysis performed, the genotypic closeness ratings were found between 0.64-0.95 frequency (Fig. 1). Among the parents used in the study, the highest closeness (0.77) was found between in Bülbül 89 (28th line) and Karatay 94 (30th line). While these two varieties have very similar characteristics, they are the types of breeding establishments that are brought to the stage by selection and whose origins may possibly be close. Because wild barley (7th line) has mushy grains, some registered varieties required 0.67 degrees; naked barley (38th line) was 0.64 degrees closer to these three parents.

When Fig. 1 is examined, it is seen that there are three main groups of closeness among the genotypes. The first group consists of 13 genotypes, and the average length of plant height and duration is 94 cm and 107 days, respectively. Within the group, there are a large number of naked grained genotypes. The closeness to the parents is 0.63, and the commonness is 0.70.

The second group consists of 16 genotypes, and the average length of plant height and duration is 98 cm and 110 days respectively. In this group where three of the parents (Bülbül 89, Karatay 94 and Wild barley) are present, the more frequent and tall genotypes are the majority. Their closeness is between 0.67-0.77 degrees for Bülbül 89 and Karatay 94 varieties, Wild barley is 0.66, naked barley is 0.64, and common affinity is 0.66.

The third group consists of 6 genotypes and the average length of plant height, and spike duration is 88 cm and 102 days respectively. In this group of parents with naked carp, early, short and bare-grain genotypes are common. The closeness to hulles barley is between 0.67-0.83 degrees, the other three parents are 0.64, and the common affinity is 0.64 degrees.

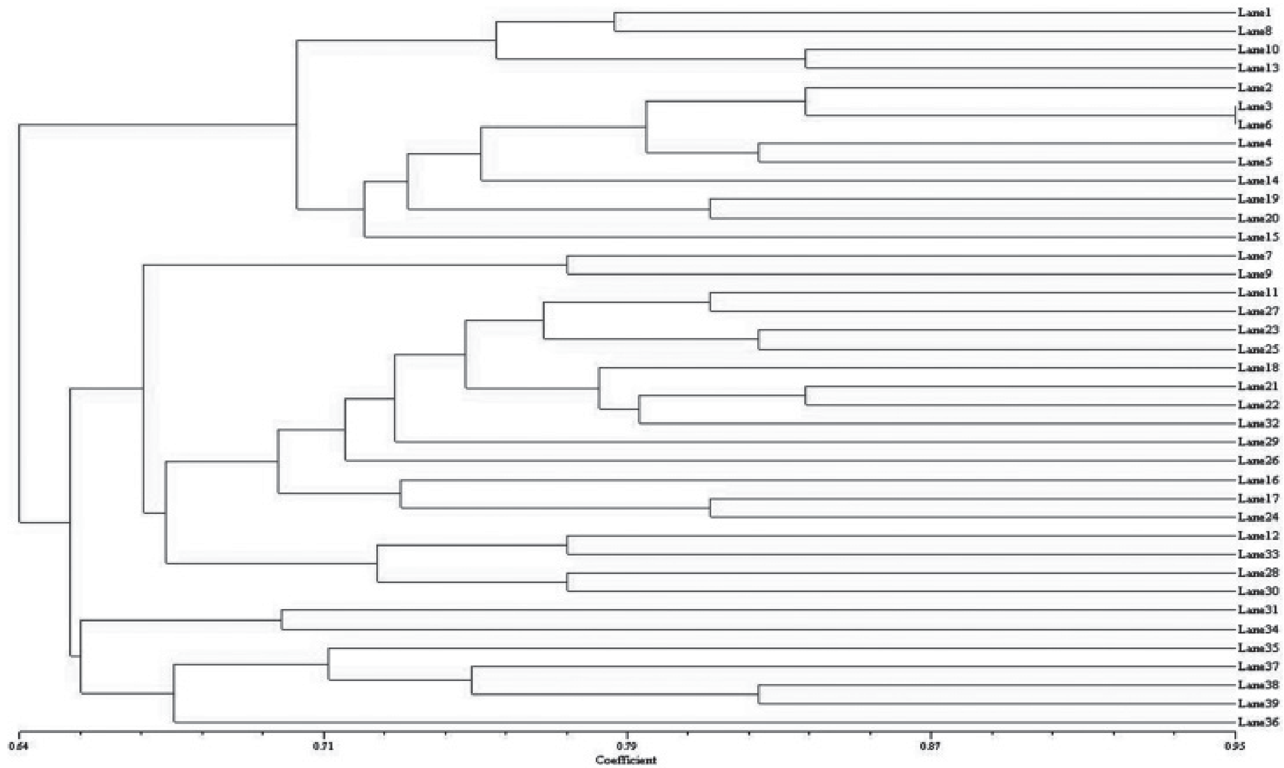

Fig. 1 ISSR Dendrogram showing the genetic relationships among 36 barley genotypes from three species

Similar results can be seen in the research conducted in this respect. In a study [15], genetic variation analysis of wild close relatives of barley collected from Tibet and the Middle East were carried out using ISSR and SSR markers. Ten ISSR primers yielded 91 allelic variants, of which 79 were polymorphic (86.81 %), in the Tibetan barleys and 82 allelic variants, of which 66 were polymorphic (80.49 %), in the Middle East genotypes. Fernandez et al. [2] can conclude that the bulk analyses of RAPD and ISSR markers were useful for studying the genetic relationships between barley cultivars, providing the ISSR markers a powerful tool for the generation of potential fingerprinting diagnostic markers for cultivars. Also, the phylogenetic analysis on the basis of ISSR-derived phenogram supports the known origin of the barley cultivars, in spite of the multiple generations of selection carried out after the ancestor crosses. According to Giancarla *et al.* [3], the genetic variability available in the germplasm of the crop solely affects the success of a breeding program. The usage of cultivars from diverse clusters and sub-clusters offer the probability of obtaining a suitable genetic variability in the crossbred population [16].

According to the results obtained, some morphological and phenological characteristics of the genotypes in the study are related to the degree of closeness to the parents. Although the hybrid genotypes are more closely related to the parents, the parental characteristics have become more dominant and prominent.

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