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USING SSR MARKERS FOR ASSESSMENT GENETIC DIVERSITY AND DETECTION DROUGHT ESCAPE CANDIDATE GENES IN BARLEY LINES (*HORDEUM VULGARE* L.)

ABSTRACT

Assessment of genetic diversity using molecular markers is one of the primary and important steps in breeding programs. In this study, genetic diversity of 52 barley lines evaluated using 68 SSR primer pairs and 47 primer pairs produced clear and polymorphic banding pattern. In general, 153 polymorphic alleles detected. The number of observed polymorphic alleles varied from 2 to 9, with an average of 3.26 alleles per locus. Polymorphic Information Content (PIC) ranged from 0.07 to 0.81, with an average of 0.45. In this research, SSR markers differentiated the studied lines efficiently. Using cluster analysis, studied barley lines divided into two groups. Genetic diversity was relatively corresponding with geographical origins, because the lines related to a country somewhat diverged from each other. Two-rowed Iranian and Chinese barleys classified in one subgroup. Also, most six-rowed barleys classified in one subgroup. Association mapping analysis was used to identify candidate genes for drought escape in barley lines and 16 informative markers were identified after which confirmation in other tests could be suitable for marker assisted breeding drought escape.

Key words: Association analysis, barley, genetic diversity, Microsatellite markers (SSR), drought escape

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INTRODUCTION

Barley (*Hordeum vulgare* L.) is a main cereal crop which is ranked as fourth crop after rice, wheat and maize in the world's food productions and second crop after wheat in Iran (FAO, 2010).

Estimations of genetic relationship and genetic diversity have always been important goal for breeders (Meszaros *et al.*, 2007), because success in breeding purposes, utilization and effective conservation of genetic resources depend on correct estimations of these parameters.

Morphological markers, storage proteins and DNA markers have been used to evaluate genetic diversity. However, due to the limited number of indicators and impact of environmental factors on morphological and proteins markers, today DNA markers are used to determine genetic diversity in plants (Zhang *et al.*, 2002). DNA markers are not influenced by environmental conditions; this is why molecular methods are appropriate alternative for morphological criteria (Pirseyedi *et al.*, 2006). Some of DNA marker techniques consist of RFLP, RAPD, SSR, AFLP and STS has its own advantages and disadvantages (Liu *et al.*, 1996). Simple sequence repeats (SSRs) markers that are based on the polymerase chain reaction (PCR) have properties like high level polymorphism, codominant inheritance, highly reproducibility, locus specificity and random distribution on the genome (Russell *et al.*, 1997) that make them superior markers for assessment of genetic diversity, genetic relationship and phylogenetic development. Information value of SSRs technique as powerful tool for genetic studies in barely breeding have been frequently confirmed in several investigations (Saghai Maroof *et al.*, 1994; Struss and Plieske, 1998; Feng *et al.*, 2003; Varshney *et al.*, 2007; El-Awady and El-Tarras, 2012; Nandha and Singh, 2014).

Association mapping has been suggested as alternative to bi-parental mapping method to detect candidate genes of interested traits in the genome. The power of Linkage disequilibrium and single marker-trait regression methods to recognition and describing loci/genes associated with economic traits have been proved in barley, maize, rice, soybean, pearl millet and wheat (Kraakman *et al.*, 2004;Wilson *et al.*, 2004; Agrama and Yan, 2007; Shi *et al.*, 2010; Kannan *et al.*, 2014; Zhang *et al.*, 2014).

The studies on drought stress effects at different growth stages of barley demonstrated that drought stress was the most pernicious during and just before spike emergence, during anthesis and initial stages of grain development. In rainfed agriculture, prolonged drought stress from heading to grain maturity reduce yield. Therefore, acceleration of heading may allow plants to complete life cycle before serious water deficit occurs (Samarah, 2005) and to escape from drought. This prevents major losses in the grain yield of rainfed crops.

In developed countries, most cereal produced in rainfed agriculture (more than 80 percent of cereal area) (Oweis *et al.*, 2009). With global climate change,

identification chromosomal regions controlling the traits related to drought stress response is a major goal in breeding programs.

In this research, we used 68 SSR markers that cover the seven chromosomes of barley to assessment genetic diversity and geographical separation of 52 lines collected from Iran, China and Egypt. In addition, genome association mapping strategies was used to detect genomic regions associated with drought escape.

MATERIALS AND METHODS

In this study, 52 barley lines of rain fed agriculture were investigated. These lines are originated from Iran (19 lines), Egypt (12 lines) and China (21 lines).

DNA was extracted from young leaves by using CTAB method (Saghai Maroof *et al.*, 1994). Qualification and quantification of extracted DNAs were determined by 0.8% agarose gel electrophoresis and spectrophotometer at 260 nm, respectively (Doulis *et al.*, 1999).

In this experiment, 68 SSR primer pairs were used for amplification of microsatellite loci. These primer pairs were selected from http://Wheat.pw.usda.gov/GG2/Barley site, which were distributed on seven linkage groups of barley.

Total reaction for DNA amplification was 10 µl, containing 25 ng/µl genomic DNA, 10 pmol/µl primers and using 1X PCR Master Kit (CinnaGen PCR Master Kit, Cat. No. PR8250C). Amplification performed in a T-Gradient Thermalcycler (BioRad, USA) with 94°C for 4 minutes, followed by 35 cycles of 94°C for 1 minute, 50 to 60°C for 55 seconds, and 72°C for 90 seconds with a final extension of 72°C for 7 minutes. To monitor polymorphisms of markers, the PCR products were loaded on 4.5% denatured polyacrylamide gel, and then visualized by silver staining (Bassam *et al.*, 1991). Also for identifying alleles, a 50-bp molecular weight ladder was used.

In field test, five traits related to drought escape including days to heading, days to grain filling, days to physiological ripeness, thousand kernel weight and yield (kg/plot) were scored on 52 barley lines. The lines were planted in a rectangular lattice with two replications in January for two years. The plot size was 0.75 m^2. The number of days counted after emerging 50% of seedlings from the soil surface. In addition to, the heading, grain filling and physiological ripeness date were recorded when 50% of plants in each plot were heading, completed grain filling and ripeness, respectively. Yield (kg/plot) was recorded by harvesting all plants in each plot.

Data scoring and Statistical analysis

The amplification fragments were scored in binary form for the present (1) or absent (0) of a band. Each of the bands assumed as one allele. The number of alleles per locus (Na), Shannon's information index (I) and principal coordinate

analysis (PCA) were calculated with GenAlEx software 6.2 (Peakall and Smouse, 2006). In analysis of molecular variance (AMOVA), genetic variance contributed into within and between groups (Excoffier *et al.*, 2005).

Polymorphic information content (PIC) and expected heterozygosity (He) was calculated as described by Wei *et al.* (2005).

$$H = 1 - \sum_{i=1}^{n} pi^2$$

$$PIC = 1 - \sum_{i=1}^{n} pi^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2\mathrm{pi}^2\, pj^2$$

where *Pi* and *Pj* is the frequency of the i^{th} and j^{th} allele in microsatellite locus, respectively. These parameters were calculated based on 1000 bootstrap samples using PowerMarker v. 3.25 (Liu, 2004).

The genetic distance of lines and cluster analysis were performed based on Maximum Composite Likelihood (Tamura *et al.*, 2011) and un-weighted pair-group method with arithmetic mean (UPGMA) algorithm by MEGA 5 software (Tamura *et al.*, 2011).

Association analysis was assessed with SPSS 19. In addition, Pearson coefficient of correlation was calculated between markers and phenotypic data. The markers that had significant correlation coefficient (5% or 1%) with each trait were used in Stepwise regression analysis for that trait. Stepwise regression analysis was used to determine informative markers on phenotypic data as dependent variable and marker data as independent variable. For prevention of type I error, probability of F test was 0.05 for entry to model and was 0.01for removal from model.

RESULTS

Criterions for genetic diversity

The relationships of the 52 barley lines were examined using 68 primer pairs and 52 primer pairs were produced clear bands. Out of 52 primer pairs, 5 primer pairs produced monomorphic banding pattern. In total, 153 polymorphic alleles (from 47 primer pairs) were detected. The number of alleles per locus ranged from 2 to 9 with an average 3.26 alleles per locus. Therefore, these set of SSR primers had good efficiency in producing polymorphic banding pattern among the studied lines and revealed suitable diversity among the lines. In addition, the PIC values were ranged from 0.07 (GBM5012) to 0.81 (Bmac0144) with an average of 0.45 (Table 1). Also, the expected heterozygosity ranged from 0.08 (GBM5012) to 0.84 (Bmac0144) with a mean of 0.52 (Table 1). The genetic diversity, often named as

expected heterozygosity, shows the probability that two alleles chosen randomly from two individuals are different (Matsuoka *et al.*, 2002). In addition to, the results showed that the lowest and highest major allele frequencies were 0.21 and 0.96 that belonged to Bmac0144 and GBM5012 markers, respectively.

Table 1

Allele number, frequency of major allele, gene diversity, polymorphic information content and chromosomal location for SSR primer pairs in barley lines

SSR primer pairs	Allele number	Frequency of major allele	Gene diversity	PIC	Chromosomal location
GBM1412	4	0.78	0.37	0.34	1H
Bmag0211	4	0.46	0.65	0.59	1H
GBM1411	2	0.89	0.19	0.17	1H
GBM1434	2	0.74	0.39	0.31	1H
GBM1281	5	0.40	0.69	0.63	2H
GBM1214	5	0.37	0.75	0.71	2H
GBM1468	2	0.55	0.50	0.37	2H
scssr08447	2	0.70	0.42	0.33	2H
GBM1280	2	0.66	0.45	0.35	3H
GBM1450	3	0.46	0.61	0.53	3H
GBM1078	2	0.64	0.46	0.35	3H
GBM1425	2	0.53	0.50	0.37	3H
scssr25691	3	0.46	0.64	0.57	3H
Bmag0121	2	0.50	0.50	0.38	3H
Bmag0508A	5	0.45	0.69	0.64	3H
GBM1242	2	0.71	0.41	0.33	3H
GBM1163	3	0.47	0.64	0.56	3H
GBM1162	2	0.50	0.50	0.38	3H
scssr20569	3	0.77	0.38	0.33	4H
BMAG0808	4	0.43	0.69	0.63	4H
GBM1452	3	0.38	0.66	0.58	4H
scssr18005	4	0.60	0.54	0.46	4H
Bmac181	2	0.95	0.09	0.09	4H
HVM68	4	0.57	0.58	0.51	4H

Table 1

Continued

SSR primer pairs	Allele number	Frequency of major allele	Gene diversity	PIC	Chromosomal location
Bmag0353	5	0.35	0.73	0.68	4H
Bmac0175	2	0.68	0.44	0.34	4H
GBM1501	3	0.69	0.47	0.41	4H
GBM1448	4	0.54	0.62	0.56	4H
HVM67	3	0.73	0.44	0.39	4H
GBM1453	3	0.56	0.52	0.42	4H
scssr02503	3	0.60	0.53	0.46	5H
HVLEU	2	0.93	0.14	0.13	5H
Bmac0144	7	0.21	0.84	0.81	5H
HvLOX	2	0.50	0.50	0.38	5H
scssr03907	7	0.35	0.76	0.73	5H
af166121	2	0.50	0.50	0.38	6H
Bmac0018	5	0.50	0.65	0.60	6H
GBM5012	2	0.96	0.08	0.07	6H
HVM65	2	0.50	0.50	0.38	6H
scssr02093	3	0.65	0.50	0.43	6H
Bmac0040	9	0.49	0.71	0.68	6H
GBM1356	2	0.50	0.50	0.38	6H
Bmac0282	2	0.58	0.49	0.37	7H
GBM1432	2	0.83	0.28	0.24	7H
EBmac0827	5	0.33	0.75	0.71	7H
Bmac167	4	0.38	0.68	0.62	7H
GBM1102	2	0.53	0.50	0.37	7H
Mean	3.26	0.57	0.52	0.45	-

Genetic diversity pattern

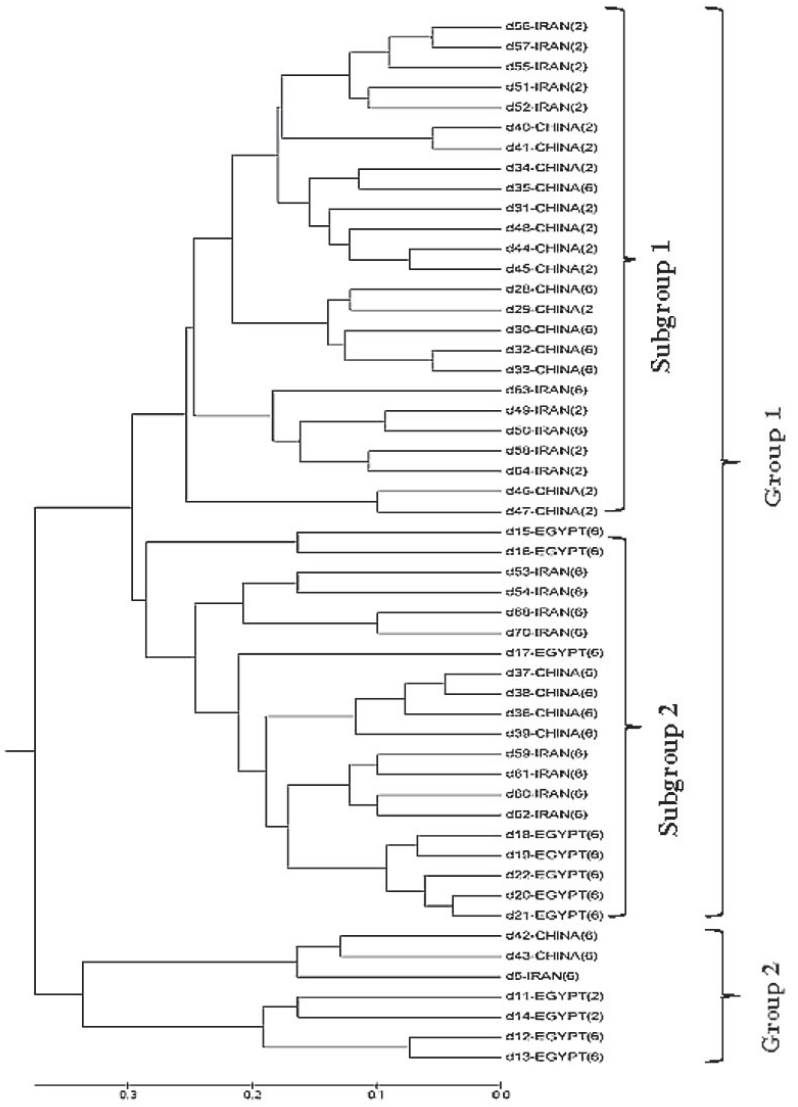

Fig. 1. Dendrogram derived from UPGMA cluster analysis using polymorphic microsatellite markers

To documentation relationship lines, genetic distance calculated and its dendrogram constructed (Fig. 1). By truncating dendrogram, 52 lines classified in two distinct groups. The first group consisted of two subgroups and the second group included one subgroup. The groups contained the lines with different geographical origin. All lines in first subgroup were belonged to Iran and China,

while the lines from Iran, China and Egypt located in second subgroup. In addition, in the second group, the lines were belonged to Iran, Egypt and China.

Most of the lines in subgroup 1 were two-rowed barley from Iran and China. However, a few of them were six-rowed Iranian and Chinese barleys, whereas all lines in subgroup 2 were six-rowed. On the other hand, in the second group the lines were two- or six-rowed barleys.

Shannon's information index represents the amount of genetic variation. In this study, the highest value was 0.768 for subgroup 1 and the least amount was 0.706 for group 2. These results, indicated gene diversity in groups. Therefore, these groups are a good source of genetic diversity.

In addition to, the private alleles were 33, 25 and 4 for subgroup 1, subgroup 2 and group 2, respectively. This set of markers is very informative, and can use to refer a new individual to a certain population or group. Using this information reduce cost and time to refer individuals to groups due to reduce number of markers.

Principle Coordinate Analysis (PCA)

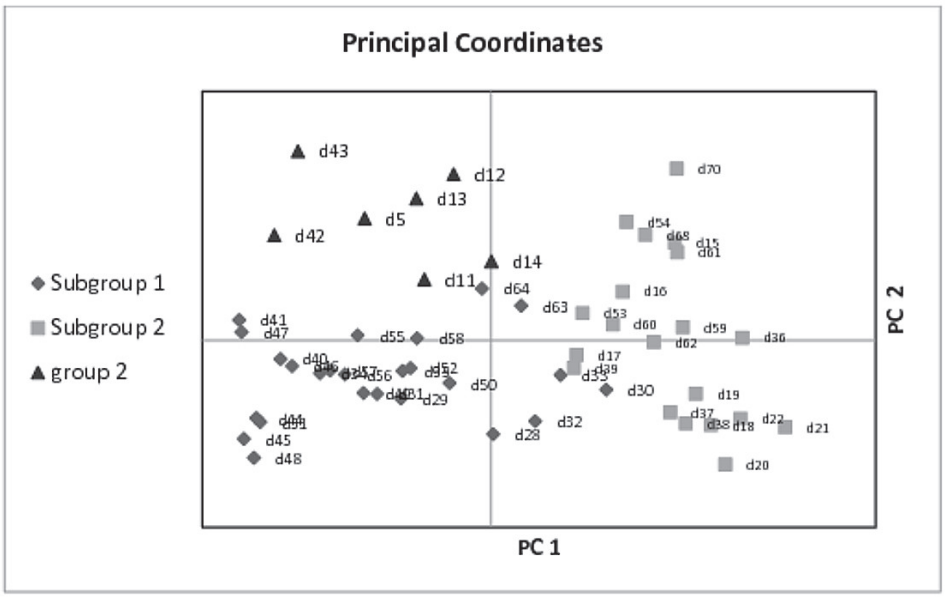

Fig. 2. Classification barely lines derived cluster analysis by the first two axes derived from Principal Coordinate Analysis

In the principle coordinate analysis (PCA), associations among the lines were assessed. The first and second axes in PCA method displayed the location of the lines in a two-dimensional plot (Fig. 2). The PC1 and PC2 legitimized 29.39% and 16.07% of total variance, respectively. The PCA results corroborated completely the results of cluster analysis based on UPGMA algorithm.

Association analysis

Association analysis was performed between the five traits related to drought escape including days to heading, days to grain filling, days to physiological ripeness, thousand kernel weight and yield with microsatellite markers and 16 informative markers identified (Table 2).

Table 2

Name, chromosomal location and regression coefficient of informative markers for traits related to drought escape in studied lines.

Trait	Informative markers	Chromosome number	Regression coefficient	Standardized regression coefficient
Days to heading	GBM1468.2	2	-2.312 ± .761	-.377
	GBM1434.1	1	-3.945 ± .660	-.562
	GBM1102.1	7	3.031 ± .801	.490
Days to physiological ripeness	Bmag0508a.4	3	-3.605 ± 1.180	-.348
	GBM1214.4	2	-3.331 ± .897	-.410
	Bmac0167.3	7	-2.193 ± .677	-.365
	scssr18005.3	4	1.768 ± .653	.299
Days to grain filling	GBM1163.1	3	-2.320 ± .747	-.343
	GBM1434.1	1	3.377 ± .842	.432
	GBM1214.2	2	-2.793 ± .788	-.406
	HVM68.3	4	-3.785 ± 1.228	-.322
Thousand kernel weight	GBM1214.2	2	-5.625 ± 1.589	-.362
	GBM1432.1	7	-10.978 ± 1.994	-.443
	GBM1468.2	2	5.790 ± 1.464	.376
Yield [kg/plot]	GBM1102.1	7	-.119 ± .016	-.790
	Bmac0144l.4	5	.057 ± .019	.322

Drought escape plays a vital role in some crop plants especially that planted in rainfed farming and is the simplest way of survival under drought conditions, because drought occurs either in the mid or late-crop season. When drought occurs late in the growth season, yield of early cereal varieties are less affected by severe drought than late maturing ones. Some traits like days to heading, days to grain filling and days to physiological ripeness determinate growth habits (early or late maturing). In this experiment, GBM1214.2 showed negative regression coefficient in days to grain filling and thousand kernel weight, since when the number of days for grain filling decrease plant have not enough time for increasing kernel weight.

On the other hand, GBM1434.1 had negative and positive regression coefficient in days to heading and days to grain filling, respectively. When the number of days to heading decrease, plant will has adequate time for grain filling before a drought occurs. However, GBM1468.2 showed positive effect on thousand kernel weight and negative effect on days to heading.

DISCUSSION AND CONCLUSIONS

In the present study, we investigated genetic diversity and genetic relationship among 52 barley lines that originated from Iran, China and Egypt using SSR markers. Hamza *et al.* (2004) believed the genotypes with same origin implicate narrow genetic diversity, because they may have exchanged genetic material through breeding programs. However, the barley lines that used in this research, were collected from three countries and had relatively broad genetic diversity.

In this experiment, the mean of PIC value was 0.45 that signified these set of SSR markers were favorably informative. Polymorphism Information Content (PIC) is a criterion for assessment of the differentiation power markers. In fact, discriminating power of a marker depends on the number of alleles and relatively frequency of these alleles (Senior *et al.*, 1998). Comparing the number of alleles and PIC showed that the markers with high number of alleles also had higher rate of PIC and were informative for population studies.

Comparison of major allele numbers indicated that the maximum frequency of major alleles was related to markers which possess fewer alleles. Therefore, locus with lower major allele frequency had more differentiation power. In general, with increasing the numbers of alleles, elevated the genetic diversity and PIC. This finding suggests the potent and direct relationship of these two parameters.

To represent relationship among lines, dendrogram was constructed by UPGMA methods and the studied lines were located in two groups. As delineated from dendrogram, the barley lines clustered partially related to geographical origin. In addition, two-rowed and six-rowed lines diverged mostly from each other and some of six-rowed and two-rowed barley lines classified in one group. This result indicates that presumably these lines originated from common ancestor(s) or exchange the genetic materials among these countries plays a vital role.

In addition to, association mapping analysis was used to identify the candidate genes for drought escape in barley lines and 16 informative markers were identified that after which confirmation in other tests could be suitable for marker assisted breeding drought escape. Furthermore, with global warming, identification suitable candidate genes for drought escape are valuable to improve rainfed farming cultivars to food security.

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