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Evaluation of genetic diversity in *Agropyron cristatum* using ISSR molecular marker

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Abstract

In order to evaluate genetic diversity, 13 accessions of *Agropyron Cristatum* were investigated using ISSR molecular markers. The 12 ISSR primers amplified a total of 65 bands of which 60 bands showed polymorphism and 5 bands were monomorphic. Maximum number of bands (8) was related to the primers IS9 and IS13, while minimum bands (4) belonged to the primers IS3 and IS12. The percentage of polymorphic bands (PPB) ranged between 60 and 100. Mean numbers of scored bands (NSB) and polymorphic bands (NPB) per primer were 5.42 and 5, respectively. The PIC values for varied from 0.23 to 0.47 with an average of 0.35. The lowest and the highest PIC indices were recorded for primers IS3 and IS6, respectively. Therefore primer IS6 determined genetic distance much better than other primers so it can be used for the analysis of genetic diversity in agropyron in the future investigations. The primers IS3 with the lowest PIC didn't have good ability to discriminate genotypes. Cluster analysis based on Dice coefficient of genetic distance classified the accessions into four groups. The first group consisted of genotypes G7 and G13 with average similarity coefficient 0.68. The second group included the accessions 4, G5, G10 and G11 with similarity coefficient 0.71. Genotypes G6, G8 and G9 were located in the third group with similarity coefficient 0.63 The fourth group had the genotypes G1, G2, G3 and G12 with similarity coefficient 0.58, therefore maximum similarity belonged to group 2, while minimum similarity was attributed to group 4. Biplot analysis divided the genotypes into 4 groups which is in accordance with results of cluster analysis. Molecular analysis of variance confirmed significant difference between groupings of accessions based on cluster analysis.

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Introduction

Plant genetic resources for food and agriculture are the basis of global food security. They comprise diversity of genetic material contained in traditional varieties, modern cultivars, crop wild relatives and other wild species. Genetic diversity provides farmers and plant breeders with options to develop, through selection and breeding, new and more productive crops, that are resistant to biotic and a biotic stresses and adapted to changing environments (Rao, 2004).

Agropyron, as a range plant grows at the most of the rangeland of Iran, which have wide adaptation and grow in different climates. Therefore, gene pool conservation and its accurate application can be used in plant breeding programs in order to improvement rangeland and increasing forage production (Arghavani *et al.,* 2010). Gene pool of *Agropyron* includes about 19 species in Iran and 150 species in the world (Bor, 1970). *Agropyron* has been applied in wide hybridization specially to transfer alien genes into cultivated wheat (Xu and Conner, 1994). Variablity among different wheatgrass (*Agropyron*) based on morphological and chemical traits were determined (Farshadfar and Farshadfar, 2004).

Genetic variability is the raw material of crop breeding industry on which selection acts to evolve superior genotypes. Before, the appearance of molecular techniques, genetic diversity was estimated from morphological features and pedigree data or agronomic traits. Estimates based on pedigree data are generally exaggerated and often unreal (Kuleung *et al.*, 2006) and morphological traits are often influenced by environmental factors (Španić *et al.*, 2012). Morphological and physiological traits are poor in number and influenced by the environmental factors (Manifesto *et al.*, 2001). Today, considerable attention has been devoted to the use of molecular markers. These markers have benefit over morphology and pedigree data for studying genetic diversity. They are not affected by the environmental effects and show genetic similarity without prior knowledge of pedigree data (Kuleung *et al.*, 2006). Molecular markers are autonomous of tissue or environmental effects, and allow cultivar identification early in plant development (Manifesto *et al.*, 2001) and also make available a direct measure of genetic diversity and go beyond the indirect diversity measures based on agronomic traits or geographic origin (Ijaz and Khan, 2009).

Inter simple sequence repeat (ISSR) is a dominant molecular marker revealed in mass. ISSR has recently been developed as an anonymous, RAPD–like approach that accesses variation in the numerous microsatellite regions dispersed throughout the various genomes and circumvents the challenge of characterizing individual loci that other molecular approaches require. They are characterized by mono-, di- or multi - nucleotide repeats that have 4 -10 repeat units side-by-side. Extremely high variability combined with greater robustness in repeatability experiments and less prone to changing band patterns with changes in constituent or DNA concentration template make them superior to other readily available marker systems in investigations of genetic variation (Fang and Roose, 1997). Genetic variations based on DNA markers for between and within different species of *Agropyron* were reported by many researchers (Refoufi, and Esnault, 2008; Szczepaniak *et al.*, 2009; Che *et al.*, 2011; Arghavani *et al.*, 2010).

Najaphy *et al.,* (2012) revealed that ISSR markers provided sufficient polymorphism and reproducible fingerprinting profiles for evaluating genetic diversity of wheat genotypes. El-Assal and Gaber, (2012) investigated the discriminating capacity of ISSR markers in establishing genetic relationship and diversity among wheat genotypes. Sofalian *et al.,* (2009) showed that ISSR markers could be efficiently used to evaluate genetic variation in the wheat germplasm. Chowdhury *et al.,* (2008) used ISSR markers for fingerprinting in a set of 27 genotypes which comprised Indian bread wheat varieties released for high yield, quality and abiotic stress and found that the cluster analysis based on molecular data is in agreement with their known origin.

Pasqualone *et al.,* (2000) found a high efficiency of ISSR markers to assess the genetic diversity and distinguish all the durum wheat cultivars examined.
The main goals of the present study were to test the efficiency of ISSR primers to measure the relationship between *agropyron cristatum* accessions and evaluate the genetic diversity among advanced genotypes for future breedin gprograms using ISSR molecular markers..

Materials and methods

Plant genetic materials

In order to evaluate genetic diversity, 13 accessions of *Agropyron Cristatum* were prepared from gene bank of the Research Institute of Forests and Rangelands, Tehran, Iran (Table 1).

DNA extraction and PCR amplification

Total genomic DNA extracted for young leaves of greenhouse-grown plants using a modified CTAB described by Doyle *et al.* (1987). Quality and quantity of extracted DNA were examined using 0.8% agarose gel. The PCR mixtures were carried out according to table 2.

Template DNA was initially denatured at 950C for 5 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 30 seconds at 950C, primer annealing for 30 seconds at the temperature based on primer temperature (temperatures of annealing in this study was 50, 55 and 60 0C) and primer extension for 1 min at 720C. A final incubation for 5 min at 720C was performed to ensure that the primer extension reaction proceeded to completion. The PCR amplified products were separated by electrophoresis on a 1.5% agarose gels using TBE buffer. The gels were put in the Ethidium bromide for 30-45 min and visualized by gel document. Numbers, codes and sequences of ISSR are presented in Table 3. *Band scoring and data analysis*

For each ISSR marker, total amplified bands, number of polymorphic bands, and percentage of polymorphic bands (PPB) were recorded. To measure the informativeness of the ISSR markers to differentiate between wheat genotypes, polymorphism information content (PIC), effective multiplex ratio (EMR), marker index (MI) and resolving power (RP) were calculated. PIC was calculated according to the formula of Anderson *et al.* (1993), as PIC = 1 - $\Sigma pi2$, where *pi* is the frequency of the *i*th allele of the locus in the set of thirty wheat genotypes. EMR is the product of the fraction of polymorphic bands and the number of polymorphic bands (9). MI was determined according to Powell *et al.* (15) as the product of PIC and EMR. RP was calculated using the formula RP=ΣIb, where Ib is band informativeness and Ib= 1-[2 × (0.5 - p)], where *p* is the proportion of genotypes containing the band (1).

ISSR bands were treated as binary characters and coded accordingly (presence =1, absence = 0). Number of bands scored, Number of polymorphic bands, Percentage of polymorphic bands were calculated for each primers and each genotypes. Marker index (MI), Polymorphism information content (PIC), effective multiplex ratio (EMR) and resolving power (RP) were measured and calculated for each primer (Anderson *et al.*, 1993). Cluster analysis, similarity matrix and principal coordinate analysis axis were carried out for 13 genotypes using NTSYSpc2.02e and GenAlex 3 (Peakall and Smouse, 2006).

Results and discussion

ISSR polymorphism

Fifteen ISSR primers were initially screened for their ability to produce polymorphic patterns across 13 agropyron genotypes. Twelve primers that were repeatable and produced high resolution bands for all the genotypes were selected for evaluation of genetic diversity in the accessions (Table 3). The 12 ISSR primers amplified a total of 65 bands in the set of 13 agropyron accessions, of which 60 bands showed polymorphism and 5 bands were monomorphic. Maximum number of bands (8) was related to the primers IS9 and IS13, while minimum bands (4)

belonged to the primers IS3 and IS12. Average number of scored bands (NSB) for each genotype across 12 primers was 27.46.

The percentage of polymorphic bands (PPB) ranged between 60 and 100 with an average of 90.14%. Mean numbers of scored bands (NSB) and polymorphic bands (NPB) per primer were 5.42 and 5, respectively (Table 4). The PIC values for the 12 primers varied from 0.23 to 0.47 with an average of 0.35. The lowest and the highest PIC indices were recorded for primers IS3 and IS6, respectively (Table 4). Therefore primer IS6 determined genetic distance much better than other primers so it can be used for the analysis of genetic diversity in agropyron in the future investigations. The primers IS3 with the lowest PIC didn't have good ability to discriminate genotypes.

Minimum MI, EMR and RP was related to the primers IS3, IS16 and IS16, while maximum MI, EMR and RP was observed for IS5, (IS5 and IS13) and IS5, respectively.

Table 1. Genotypes under investigation.

Number	Species	Origin	Gene bank Code
1	*Ag. cristatum*	Isfahan	619m
2	*Ag. cristatum*	Bejnord	1550
3	*Ag. cristatum*	Gorgan	1727m
4	*Ag. cristatum*	Arak	2854
5	*Ag. cristatum*	Isfahan	4056m
6	*Ag. cristatum*	Bejnord	3029
7	*Ag. cristatum*	Gorgan	1722m
8	*Ag. cristatum*	Isfahan	4045p1
9	*Ag. cristatum*	Exotic	529
10	*Ag. cristatum*	Isfahan	619p13
11	*Ag. cristatum*	Gorgan	1727p10
12	*Ag. cristatum*	Baft	7844
13	*Ag. cristatum*	Chadgan	4049

Average MI, EMR and RP was 1.75, 4.55 and 3.58, respectively.

Table 2. Compounds of optimized ISSR reaction.

To provide 20 µl	Compounds of a sample
12.6 µl	Water distillated twice
2 µl	Buffer PCR (X10)
1.5 µl	MgCl2 (50 mmol)
0.4 µl	Nucleotides mixture (10 mmol)
1.2 µl	Primer (10 µmol)
0.3 µl	Tag polymerase
2 µl	DNA (10 ng)
20 µl	total

Dice coefficient of genetic similarity

Dice coefficient of genetic similarity (Table 5) among the accessions, varied between 0.38 to 0.80. Average similarity between the genotypes was 0.57, therefore genetic variation among the entries was relatively desirable. The highest genetic similarity was between genotypes G2 and G3, while the lowest similarity observed between genotypes G5 and G13.

Hierarchical clustering

Cluster analysis based on Dice coefficient of genetic distance classified the accessions into four groups (Fig. 1). The first group consisted of genotypes G7 and G13 with average similarity coefficient 0.68. The second group included the accessions 4, G5, G10 and G11 with similarity coefficient 0.71. Genotypes G6, G8 and G9 were located in the third group with similarity coefficient 0.63 The fourth group had the genotypes

G1, G2, G3 and G12 with similarity coefficient 0.58, therefore maximum similarity belonged to group 2, while minimum similarity was attributed to group 4.

Table 3. Number, code and sequence of ISSR under investigation.

Numbers	Codes	Sequences
P1	IS3	5' GAGAGAGAGAGAGAGAYC 3'
P2	IS5	5' AG AG AG AG AG AG AG AGC 3'
P3	IS6	5' CACACACACACACACAG 3'
P4	IS7	5' GTGTGTGTGTGTGTGTC 3'
P5	IS9	5' CTCTCTCTCTCTCTCTG 3'
P6	IS10	5' GAGAGAGAGAGAGAGARC 3'
P7	IS11	5' ACACACACACACACACC 3'
P8	IS12	5' TGTGTGTGTGTGTGTGG 3'
P9	IS13	5' AGAGAGAGAGAGAGAGYT 3'
P10	IS14	5' GACAGACAGACAGACA 3'
P11	IS15	5' GGATGGATGGATGGAT3'
P12	IS16	5'DBDACACACACACACACA3'

Biplot analysis

Biplot analysis based on the the first and second coordinates (Fig. 2) divided the genotypes into 4 groups which is in accordance with results of cluster analysis. The variation explained by the first two coordinates was 31.51% and 16.27%.

Molecular analysis of variance

Analyses of molecular variance was performed for ISSR bands to determine the significant difference between groupings of accessions based on cluster analysis (Table 6). The results showed a significant ($P<0.01$) difference between groups and the portion of variance percentage for between group and within group was 24% and 76%, respectively. Genetic variation between populations of *Ag.Cristatum* was reported by Che *et al.* 2007 based on molecular variance analysis using SSR markers.

Table 4. Genetic diversity indices in agropyron cristatum using ISSR marker.

Primer code	NSB	NPB	PPB	PIC	MI	EMR	RP
IS3	4	3	75.00	0.23	0.69	2.25	1.23
IS5	8	8	100.00	0.43	3.43	8.00	7.38
IS6	4	4	100.00	0.47	1.87	4.00	3.69
IS7	5	5	100.00	0.36	1.82	5.00	2.46
IS9	5	5	100.00	0.36	1.78	5.00	2.92
IS10	6	5	83.33	0.33	1.66	4.17	5.38
IS11	6	6	83.33	0.43	2.13	4.17	4.62
IS12	4	4	100.00	0.37	1.47	4.00	2.31
IS13	8	8	100.00	0.32	2.56	8.00	4.92
IS14	5	4	80.00	0.36	1.46	3.20	4.31
IS15	5	5	100.00	0.28	1.40	5.00	2.77
IS16	5	3	60.00	0.26	0.78	1.80	0.92
Mean	5.42	5.00	90.14	0.35	1.75	4.55	3.58

Table 5. Similarity matrix of the genotypes investigated using Dice coefficient of similarity and ISSR markers.

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13
G1	1.00												
G2	0.75	1.00											
G3	0.72	0.80	1.00										
G4	0.59	0.68	0.65	1.00									
G5	0.56	0.68	0.58	0.71	1.00								
G6	0.49	0.57	0.52	0.50	0.47	1.00							
G7	0.51	0.52	0.53	0.64	0.49	0.54	1.00						
G8	0.67	0.68	0.66	0.63	0.67	0.60	0.64	1.00					
G9	0.66	0.60	0.75	0.57	0.62	0.62	0.53	0.69	1.00				
G10	0.64	0.59	0.63	0.56	0.64	0.46	0.45	0.61	0.60	1.00			
G11	0.56	0.53	0.55	0.58	0.46	0.55	0.45	0.41	0.50	0.57	1.00		
G12	0.69	0.71	0.64	0.56	0.52	0.49	0.47	0.58	0.56	0.51	0.43	1.00	
G13	0.54	0.58	0.50	0.48	0.39	0.50	0.68	0.48	0.43	0.45	0.53	0.55	1.00

Efficiency of ISSR primers were reported by other researchers to determine of genetic diversity between and within different plant species (Xu *et al.*, 2012; Hu *et al.*, 2011).

Table 6. Molecular analysis of variance using ISSR markers.

S.O.V	DF	SS	MS	Estimated variance	Percentage of variance	PhiPT	P-value
Between accessions	3	56.103	18.701	2.923	24%	0.237	0.010
Within accessions	9	84.667	9.407	9.407	76%		
Total	12	140.769		12.330	100%		

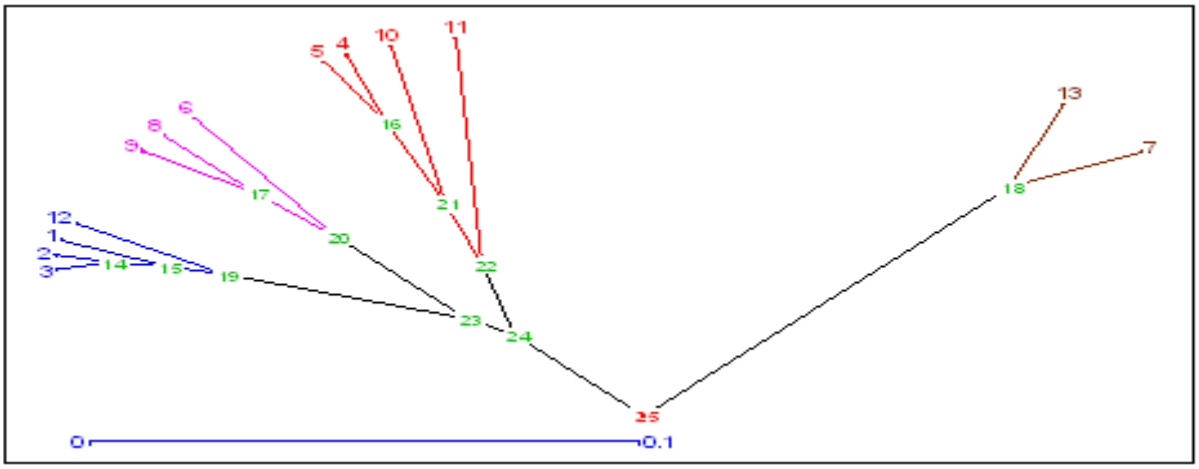

Fig. 1. Dendrogram resulted from cluster analysis of genotypes based on Dice coefficient of genetic distance using ISSR markers.

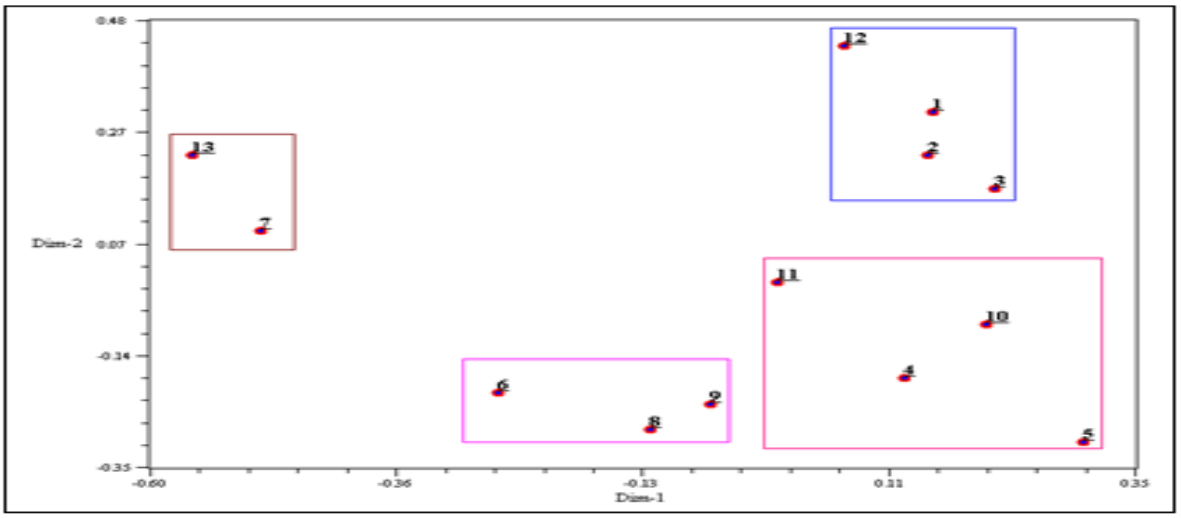

Fig. 2. Biplot of grnotypes base on first two PCA using principal coordinate analysis.

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