Efficiency of anchored and non-anchored ISSR markers to estimate genetic diversity among bread wheat cultivars

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Abstract

DNA markers are integrally connected to the success of molecular breeding and are fundamentally required by breeders to be able to, a. identify new gene sources in the available biodiversity, b. select parents in order to increase heterosis, c. decrease the number of backcross generations for gene introgression breeding programs, and d. carry out marker-assisted selection (MAS). The present research was conducted to examine the efficiency of anchored and non-anchored ISSR molecular markers for grouping 20 bread wheat cultivars introduced in a cold and moderate area of Iran. The results showed an average polymorphism of 82.69% and 75% for anchored and non-anchored ISSR markers, respectively. This result indicates that the anchored ISSR markers have a higher rate of efficiency compared to non-anchored ISSR markers. Therefore, simultaneous grouping of cultivars with the use of anchored and non-anchored ISSR markers can differentiate the cultivars of cold and moderate regions. When anchored and non-anchored ISSR markers were simultaneously applied for cluster analysis and grouping of the individuals, the pattern of genetic diversity aligned with the pattern of geographical distribution, and the cultivars attributed to identical geographical regions were allocated in one group. Based on applied clustering with two anchored and non-anchored markers, it was concluded that Pishtaz and Mahdavi cultivars had less genetic similarity compared to other cultivars.

Key words: Clustering, ISSR, Molecular marker, Wheat.

INTRODUCTION

Wheat is the staple food of about 35% of the world population and most preferred cereals in the world (Kumar *et al*., 2016), supplying nearly 55% of the carbohydrates consumed worldwide and is the second most important food crop in developing countries after rice. A primary concern of modern plant breeding is that genetic diversity has decreased during the past century. Maintenance and availability of genetic diversity is necessary for preventing disease epidemics (Ramshini *et al*., 2016). Also in recent years, the increase of wheat yield has stopped due to less diversity that exists among wheat cultivars for the plant breeding programs (Gupta *et al*., 1999).

Nevertheless, molecular markers have emerged as a fascinating technology for evaluating genetic diversity (El-Aziz *et al*., 2016), genomic fingerprinting (Kumar *et al*., 2016), gene tagging, molecular phylogenetic, genetic fidelity, disease resistance and sex determination in a wide range of crop plant species (Sharma *et al.,* 2008). In this respect, inter-simple sequence repeat (ISSR) markers have been revealed as novel DNA markers that can be very useful for the research fields which aim for crop improvement due to their high reproducibility. Although ISSR markers have

similar advantages to RAPD markers, as one of the representative low cost DNA markers, ISSR markers are more reliable than RAPD ones, due to their both higher repeatability and comparatively more length of the primers, enabling them to higher annealing temperatures (Tarinejad 2013a).

The study of genetic diversity of crop plants is essential for the identification of genetic potentials and their use in breeding programs. The use of cultivars with suitable genetic distance as parents will result in the production of hybrids with high potential (Tarinejad 2013b). Inter-simple sequence repeat (ISSR) technique is a PCR based method that involves the amplification of the DNA segment presented at an amplifiable distance between two identical microsatellite repeat-regions oriented in opposite directions. In this technique, microsatellites of normally 16–25 bp length are used as primers in a single primer PCR reaction, targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide. The utilized primers can be either unanchored (Gupta *et al.,* 1994; Wu *et al.,* 1994) or far commonly anchored at 3'or 5' end with 1 to 4 degenerate bases extended into flanking sequences (Zietkiewicz *et al.,* 1994).

Additionally, the extent of polymorphism differs both in essence (unanchored, 3'-anchored, or 5'-anchored) and the sequence of the repeats (motif) in the employed primer. When the unanchored (i.e. only the SSRs) is used as primer, it tends to slip within the repeat units during the amplification, which leads to smears instead of clear bands. Whereas, extending the primer (anchoring) with 1 to 4 degenerate nucleotides at the 3' end or 5' end can assure that annealing will be postponed until the ends of a microsatellite in template DNA and consequently will obviate internal priming and smear formation. Additionally, the anchor allows only a subset of the microsatellites to serve as priming sites. When 5'-anchored primers are used, the amplified products include the microsatellite sequences and their length varies across a genome, therefore gives more numbers of bands and higher degrees of polymorphism (Pradeep Reddy *et al.,* 2002).

It is noteworthy that di-nucleotide repeats, anchored either at 3' or 5' end, usually reveal high polymorphism and the primers anchored at 3' end give clearer banding pattern compared to those anchored at 5' end. Since the primer is a SSR motif, the frequency and distribution of the microsatellite repeat motifs vary in different species and influence the generation of bands as well. In general, primers with (AG), (GA), (CT), (TC), (AC), (CA) repeats show higher polymorphism than primers with other di-, tri- or tetra-nucleotide repeats. (AT) repeats are the most abundant di-nucleotides in plants but the primers based on (AT) would self- anneal and do not amplify (Blair *et al.,* 1999; Joshi *et al.,* 2000; Pradeep Reddy *et al.,* 2002).

In the present research, we used the dominantly expressed multilocus DNA markers, and inter-simple sequence repeats which had been successfully used in advance for cereals in genetic variation assessment, cultivar and genotype identification, fingerprinting of interspecific hybrids and gene tagging of interesting agronomic traits. The objectives of the current research can be summed up as (1) to investigate the efficiency of anchored and non-anchored ISSR molecular markers to estimate the genetic diversity among 20 bread wheat cultivars derived from two populations; (2) to determine the difference between anchored and non-anchored ISSR markers for the grouping of the cultivars; (3) to identify the location of cultivars on the axis of principal component; and (4) to assess the genetic variation patterns with geographical distribution.

MATERIALS AND METHODS

Plant material
Two populations of bread wheat cultivars (each population composed of 10 cultivars) which were introduced and released during 2006-2011, in cold and moderate areas of Iran, were cultivated (Table 1). The harvested young leaf samples were dipped into liquid nitrogen after being wrapped in an aluminum foil and stored at -20 °C.

DNA extraction method
DNA extraction was performed by using CTAB method (Saghai-Maroof *et al.,* 1984). Both the quality and quantity of the extracted DNA were determined by spectrophotometry techniques and agarose gel electrophoresis.

Polymerase chain reaction (PCR)
Six ISSR primers were used to evaluate the cultivars (Table 2). The master mix, including Taq DNA polymerase, $MgCl_2$, PCR buffer and dNTPs, was provided. Then, 10 µl of master mix was added to PCR tubes, and 1 µg/µl template DNA, 0.5 µM ISSR primer and 13.5 µl distilled water were also added (final volume of reaction was 25 µl). The initial denaturation temperature was set at 95 ˚C for 4 minute and 35 cycles of 94 ˚C, for 45 s; annealing temp at various temper-atures (Table 2), for 45 s; and extension at 72 ˚C, for 1 min. The final extension was set at 72 ˚C, for 10 min.

Table 1. The name of cultivars and populations used in the present experiment. All cultivars belong to cold and moderate area of Iran, based on the released region.

Populations	
Cultivars of cold area	Cultivars of moderate area
ALVAND	MARVDASHT
ALAMOUT	AZADI
BEZOSTAYA	SHIRAZ
MV17	KARAJ1
NAVID	NIKNAZHAD
ZARRIN	TAJAN
FALAT	KARAJ3
C854	MAHDAVI
C810	PISHTAZ
SHAHRYAR	BAHAR

Table 2. ISSR primers were used in this research.

Primer No.	Sequence of primer	Type of primer	Annealing temperature
1	5′GAGAGAGAGAGAGAGAC3′	Anchored at 3' end	48.5
2	5′GAGAGAGAGAGAGAGA3′	Non anchored	46
3	5′CACCACCACCACCACG3′	Anchored at 3' end	44.5
4	5′CACCACCACCACCAC3′	Non anchored	41
5	5′CCACCACCACCACCAC3′	Anchored at 3' end	55.5
6	5′CCACCACCACCACCA3′	Non anchored	52

To be able to detect the amplified fragments, PCR products were analyzed using agarose (1.2 % w/v) gel electrophoresis stained with ethidium bromide and only well-separated bands with high intensities were selected. Amplified products were scored based on the presence or absence of ISSR markers.

Scoring of bands

The amplified bands were scored for the presence (1) or the absence (0) of bands. To detect the size of the amplified fragments a molecular size marker with sizes of 250 -10000 bp was run along side the samples. Statistical analysis was performed by means of the GenAlEx6 software. Additionally, molecular variance analysis was applied on bias squared Euclidian distance to separate the total molecular variance among and within the cultivars. Cluster analysis was generated to demonstrate the genetic relationships of accessions using un-weighed pair-group method, arithmetic averages (UPGMA) and simple matching coefficient. The SPSS19 software was used for the cluster analysis.

RESULTS AND DISCUSSION

The comparison and contrast of the Polymorphism of Anchored and Non-anchored ISSR Markers

As illustrated in Table 3, the highest polymorphism of anchored markers was observed in the winter wheat population by 84.62%, whereas the lowest polymorphism was observed in the facultative population by 80.77%. The total number of polymorphic bands in the winter population was higher than the facultative wheat population. The lowest number of band was obtained by non-anchored Primer 2 in both winter and facultative wheat populations. The highest polymorphism of non-anchored markers was observed in the facultative wheat population by 80.77% and the lowest observed in the winter population by 69.23%. The total number of polymorphic bands was higher in the facultative population compared to the winter wheat population (Table 3). Based on these findings, the attained average polymorphism for anchored and non-anchored markers was 82.69% and

Table 3. The comparison of the polymorphism of anchored and non-anchored ISSR markers among different genotypes of bread wheat.

	Anchored Primer			Non-anchored Primer		
Primer No.	Primer sequence	Winter wheat genotypes	Facultative wheat genotypes	Primer Sequence	Winter wheat genotypes	Facultative wheat genotypes
1	GAGAGAGAGAGAGAGAC	5	7	GAGAGAGAGAGAGAGA	7	8
2	CACCACCACCACCACG	6	6	CACCACCACCACCAC	3	4
3	CCACCACCACCACCAC	7	6	CCACCACCACCACCA	7	7
Total number of polymorphic bands		18	17		17	19
Average polymorphic bands		6	5.6		5.6	6.3
Percentage of polymorphism		84.62	80.77		69.23	80.77
Average polymorphism percentage		82.69			75	

75%, respectively, that indicates better efficiency of anchored markers in polymorphism studies compared to their non-anchored counterparts.

Estimation of genetic distance among wheat genotype populations

The distance of cultivars was estimated based on Nei's (1972) genetic distance. Table 4 shows the matrix of genetic similarity between cultivars for anchored and non-anchored ISSR markers. For non-anchored markers, the highest genetic similarity was observed in the cultivars Shahryar and MV17 (0.94), Falat and Navid (0.91), Mahdavi and Shahryar (0.91), whereas the lowest genetic similarity was found in Pishtaz and Alamout (0.29), Zarrin and Pishtaz (0.27), Bahar and C810 (0.27), and Pishtaz and Niknazhad (0.24). For anchored ISSR markers, the highest genetic similarity was detected in cultivars of Marvdasht and Azadi (0.91), Tajan and Bahar (0.96) whereas, the lowest genetic similarity was found in cultivars of Alamout and Pishtaz (0.38), Mahdavi and C854 (0.35), and Pishtaz and Shahryar (0.36).

The similarity and distance among cultivars were calculated according to Nei's (1972) genetic distance after scoring the bands (The information is not presented here). The highest genetic similarity was among cultivars of Zarrin and Alamout (0.86), Karaj 1 and C854 (0.85), Karaj 1 and C810 (0.85), and Azadi and Marvdasht (0.85); the lowest genetic similarity was found in Pishtaz genotype and Alamout, Bezostaya, Marvdasht, Azadi and Mahdavi cultivars. This means that Pishtaz genotype shows the highest genetic difference compared to the rest of the cultivars.

Clustering bread wheat cultivars utilizing anchored and non-anchored ISSR markers

Figure 1A illustrates the results obtained from the clustering of different bread wheat cultivars, using non-anchored ISSR markers, according to integrating the farthest neighbor method based on Dice's similarity matrix. Dendrogram cutting from a distance of 18 of Dice's similarity matrix divided the wheat cultivars into three groups; group 1 included cultivars of MV17, Shahryar, Marvdasht and C810, group 2 included C854 and Pishtaz, and group 3 included the remaining cultivars. Such grouping indicates that cultivars from different geographical regions can also be placed in one cluster.

Figure 1B illustrates the results obtained from the clustering of different bread wheat cultivars using anchored ISSR markers with farthest neighbor method based on Dice's similarity. Dendrogram cutting from a distance of 18 allocated the wheat cultivars into three clusters. Groups 1, 2 and 3 encompassed cultivars Mahdavi; Falat, Karaj 3, C854, MV17, Pishtaz; and other cultivars. This grouping also indicated that cultivars from different geographical regions can be placed in one cluster. Therefore, the pattern of genetic diversity is not compatible with the pattern of geographical distribution, so that the cultivars cultivated in various geographical regions were situated in an identical group; for example, the cultivars associated with cold and temperate regions were located in the third cluster. Different studies have reported the incompatibility of genetic diversity with geographical diversity (Al- Rawashdeh 2011; Schuster *et al.,* 2009).

Figure 1C illustrates the results derived from the clustering of different bread wheat cultivars by anchored and non-anchored ISSR markers by using farthest neighbor method based on the Dice's similarity coefficient. Dendrogram cutting from a distance of 14

Table 4. The matrix of similarity among 20 bread wheat genotypes revealed by non-anchored ISSR markers (lower diameter) and anchored ISSR Markers (upper diameter).

	ALVAND	ALAMOUT	BEZOSTAYA	MV17	NAVID	ZARRIN	FALAT	C854	C810	SHAHRYAR	MARVDASHT	AZADI	SHIRAZ	KARAJ1	NIKNAZHAD	TAJAN	KARAJ3	MAHDAVI	PISHTAZ
ALVAND	1	0.65	0.71	0.60	0.65	0.69	0.79	0.77	0.88	0.60	0.80	0.88	0.83	0.87	0.75	0.77	0.77	0.64	0.47
ALAMOUT	0.76	1	0.81	0.64	0.80	0.87	0.77	0.55	0.73	0.71	0.85	0.86	0.70	0.80	0.67	0.70	0.70	0.72	0.29
BEZOSTAYA	0.78	0.70	1	0.79	0.80	0.80	0.77	0.69	0.80	0.71	0.79	0.87	0.72	0.64	0.87	0.64	0.72	0.74	0.38
MV17	0.74	0.67	0.69	1	0.77	0.62	0.74	0.64	0.69	0.50	0.62	0.74	0.69	0.81	0.69	0.69	0.69	0.40	0.40
NAVID	0.83	0.76	0.87	0.74	1	0.79	0.76	0.74	0.86	0.62	0.84	0.92	0.69	0.83	0.77	0.85	0.77	0.79	0.47
ZARRIN	0.73	0.84	0.76	0.72	0.79	1	0.76	0.59	0.79	0.67	0.84	0.91	0.67	0.73	0.75	0.83	0.75	0.77	0.27
FALAT	0.82	0.74	0.76	0.80	0.82	0.80	1	0.79	0.85	0.67	0.69	0.82	0.67	0.82	0.75	0.75	0.69	0.69	0.40
C854	0.70	0.59	0.53	0.52	0.60	0.44	0.56	1	0.81	0.83	0.67	0.50	0.55	0.50	0.53	0.55	0.50	0.42	0.46
C810	0.57	0.44	0.50	0.67	0.57	0.42	0.63	0.47	1	0.69	0.67	0.48	0.43	0.48	0.60	0.61	0.43	0.56	0.57
SHAHRYAR	0.76	0.69	0.79	0.94	0.83	0.74	0.74	0.56	0.62	1	0.69	0.83	0.71	0.83	0.71	0.71	0.83	0.91	0.45
MARVDASHT	0.69	0.61	0.72	0.90	0.77	0.67	0.75	0.45	0.70	0.69	1	0.90	0.77	0.92	0.67	0.77	0.71	0.80	0.42
AZADI	0.75	0.86	0.87	0.74	0.92	0.91	0.82	0.50	0.48	0.83	0.90	1	0.88	0.82	0.86	0.85	0.76	0.86	0.35
SHIRAZ	0.85	0.70	0.72	0.69	0.69	0.67	0.67	0.55	0.43	0.71	0.77	0.57	1	0.78	0.57	0.72	0.73	0.85	0.32
KARAJ1	0.87	0.80	0.64	0.81	0.83	0.73	0.82	0.50	0.48	0.83	0.92	0.82	0.78	1	0.76	0.78	0.71	0.71	0.38
NIKNAZHAD	0.75	0.67	0.87	0.69	0.77	0.75	0.75	0.53	0.60	0.71	0.67	0.86	0.57	0.76	1	0.76	0.76	0.69	0.24
TAJAN	0.77	0.70	0.64	0.69	0.85	0.83	0.75	0.55	0.61	0.71	0.77	0.85	0.72	0.78	0.62	1	0.69	0.67	0.42
KARAJ3	0.77	0.70	0.72	0.69	0.77	0.75	0.69	0.50	0.43	0.83	0.71	0.73	0.64	0.71	0.79	0.79	1	0.76	0.67
MAHDAVI	0.64	0.72	0.74	0.40	0.79	0.77	0.69	0.42	0.56	0.91	0.80	0.86	0.85	0.71	0.69	0.67	0.75	1	0.38
PISHTAZ	0.47	0.29	0.38	0.40	0.47	0.27	0.40	0.46	0.57	0.45	0.42	0.35	0.32	0.38	0.24	0.42	0.67	0.38	1
BAHAR	0.56	0.64	0.67	0.64	0.72	0.70	0.61	0.48	0.27	0.73	0.59	0.80	0.67	0.58	0.72	0.67	0.74	0.83	0.33
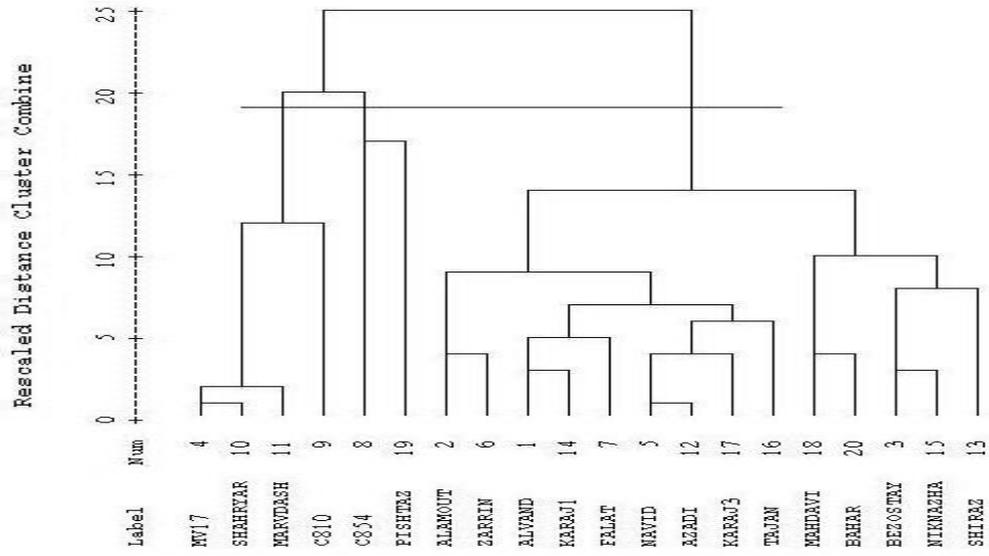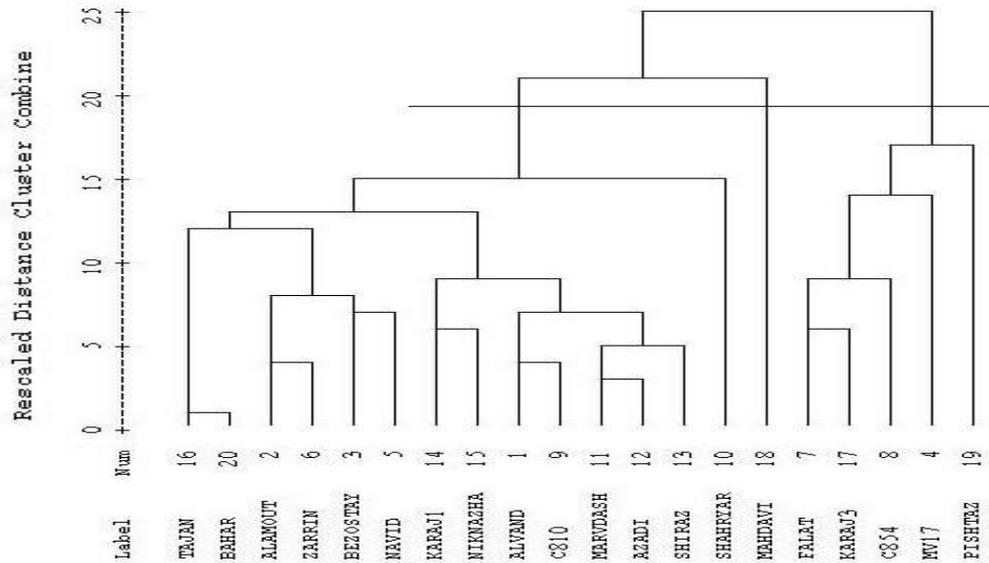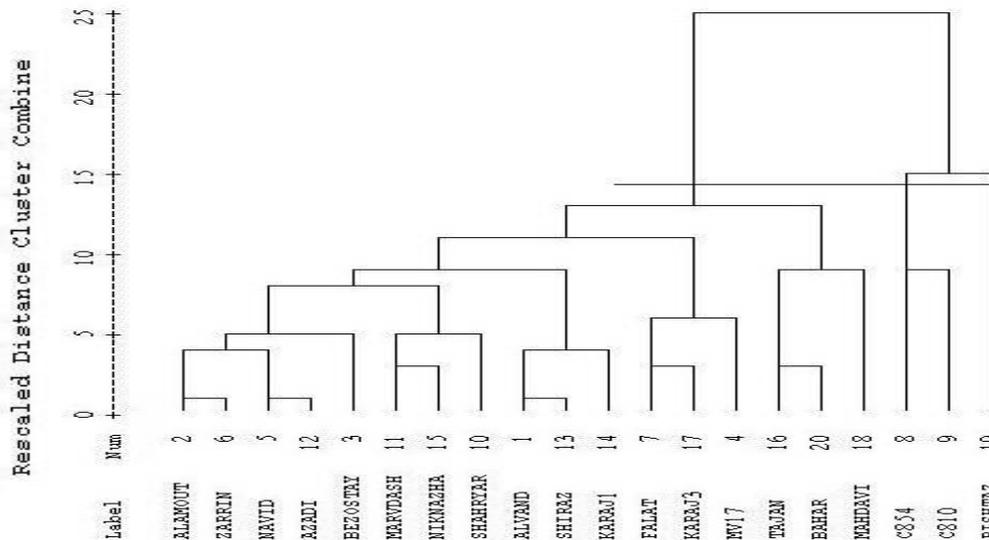
Figure1. Clustering of bread wheat cultivars by using **A:** non anchored, **B:** anchored and **C:** both ISSR markers.

of Dice's similarity allocated the wheat cultivars into three clusters. Pishtaz cultivar was placed in Group 1, two cultivars of cold regions (i.e. C810 and C854) were placed in group 2, and the others were placed in group 3. This grouping almost associated the cultivars of similar geographical regions with an identical cluster. Thus, the pattern of genetic diversity partially matches with the pattern of geographical distribution. For instance, despite the fact that the cultivars of cold and temperate regions are both in Group 3, regarding the sub-cluster of the third one, the cultivars of cold regions, namely Alamout, Zarrin, Navid and Bezostaya were allocated into one sub-cluster. Therefore, it apparently seems reasonable to use anchored and non-anchored markers simultaneously for grouping cultivars. Indeed, it should be noted that in the present study only six anchored and non-anchored markers were used. If more markers had been used, the results would have possibly been more reliable.

Principal component analysis of ISSR Markers
Principal component analysis was conducted by the application of similarity matrix and GenAlEx6 software (Figure 2). Respecting the anchored ISSR markers, the first two components accommodated 56.81% of the initial changes (Table 5). Based on the first two components, the grouping of the cultivars was carried out in a two dimensional graph (Figure 2A). As it is observed, the cultivars of cold and moderate regions were marked with blue and red signs, respectively. There was no particular pattern observed in the cultivars regarding the compatibility of genetic diversity and geographical distribution. Although no specific correspondence was observed between the results of the cluster analysis and principal component analysis, the cultivars of different cultivars were distributed on the right and left sides of the two-dimensional axes. Moreover, Mahdavi cultivar was located at the bottom of the axis, distinct from the rest.

Considering the non-anchored ISSR markers, the first two components accommodated 59.26% of the initial changes. Based on the first two components, grouping the cultivars were administrated in a two dimensional graph (with the results of this grouping illustrated in Figure 2B). As it is shown in Figure 2B, the cultivars of cold and moderate regions were marked with blue and red signs, respectively. No particular correspondence was observed between the cultivars in terms of genetic diversity and geographical distribution. Additionally, the cultivars with different geographical regions were placed on a single axis, and a particular correspondence was observed between the results of the cluster analysis and principal component analysis. The

cultivars of different clusters were distributed in the four dimensions of the axis.

By the simultaneous use of anchored and non-anchored markers, the first two components accommodated 47.61% of the initial changes. Based on the first two components, the grouping of the cultivars was carried out in a two dimensional graph (Figure 2C). The cultivars of cold and moderate regions were marked with blue and red signs, respectively. A specific corresponding pattern was observed between the cultivars, regarding the genetic diversity and geographical distribution. The cultivars of the same geographical regions were partly located on the single side of the axis; therefore, a certain correspondence was observed between the results of the cluster analysis and principal component analysis. The results indicated that genotype grouping by simultaneous use of anchored and non-anchored markers can well separate the cultivars of cold regions from those of the moderate regions.

Bornet and Branchard (2004) used 18 ISSR primers to identify Brassica species. They reported that non-anchored and anchored ISSR were suitable tools to study genetic relationships. Bornet and Branchard (2001) used four non-anchored oligonucleotide primers, namely, $(CAG)_5$, $(CAA)_5$, $(GACA)_4$, and $(GATA)_4$ and declared that polymorphisms were abundant among 7 dicot species tested by two tri-nucleotide and two tetra-nucleotide primers. Thus, non-anchored ISSR markers are a better choice for DNA fingerprinting. The results of bias markers used in this study confirmed that ISSR marker is a suitable tool to estimate genetic diversity among cultivars.

Tarinejad (2013a) used seven ISSR markers for the evaluation of the genetic distance and the grouping of thirty winter wheat cultivars. Seven ISSR primers yielded 60 bands which were 86.67 % polymorphism. The mean of heterozygosity was different among the produced alleles, varying from 0.499 to 0.033. The highest genetic distance (0.657, 0.655, 0.61 and 0.643) was observed in cultivars C-83-3/C-89-1, C-84-4/C-89-3, C-83-1/C-88-5, and C-83-4/C-83-2. Cluster analysis assigned the studied lines into four main groups via the complete linkage method based on the Dice similarity coefficient. Generally, 76.67% of total variance among data was shown by the four principle components and a good match was observed in the result of cluster analysis and PCA for grouping of cultivars: that the classification of closely related cultivars could be effective by using ISSR marker.

Sofalian *et al.* (2008) used Inter-simple sequence
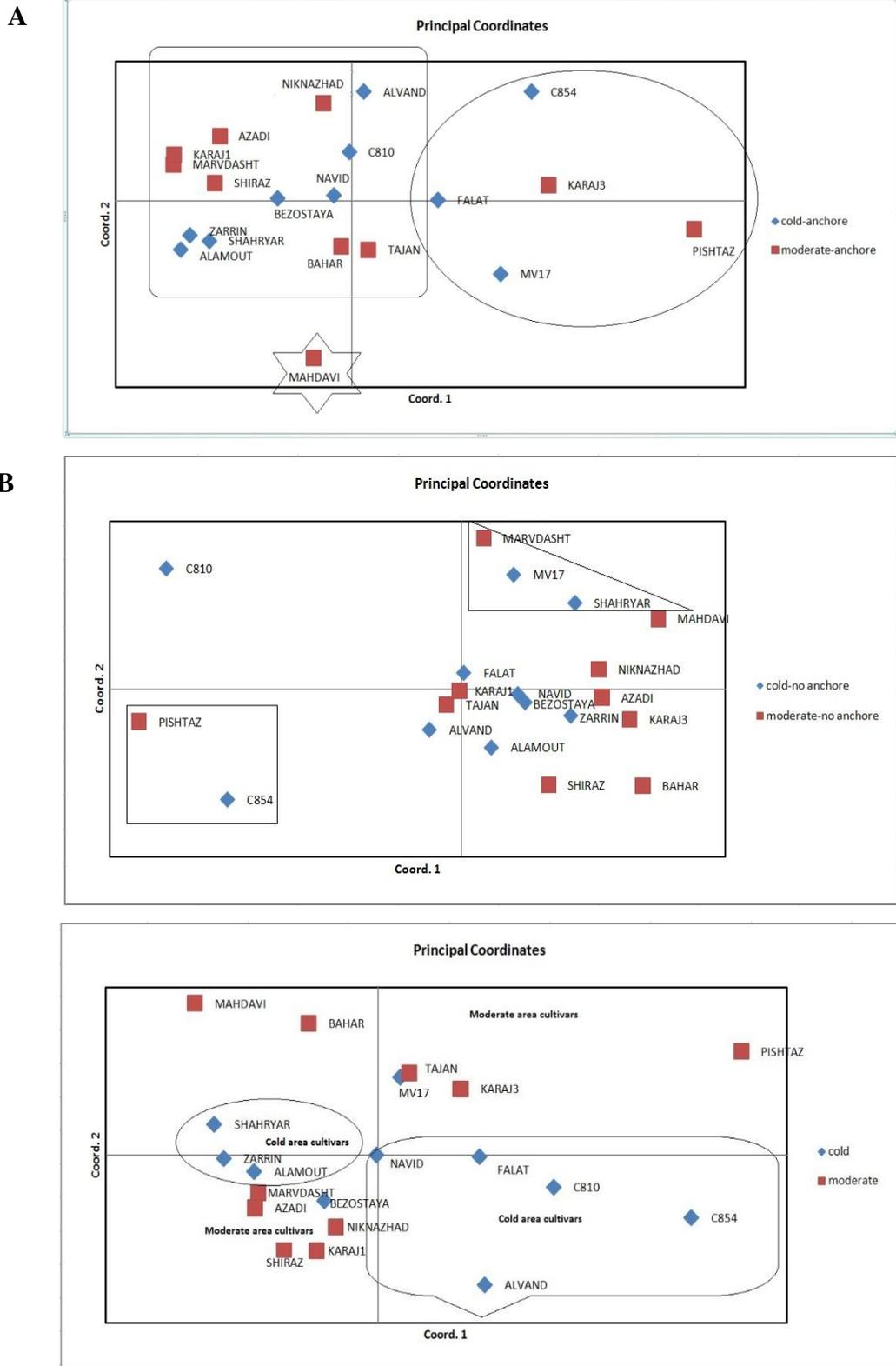
Figure 2. The grouping of different bread wheat cultivars using the two first components of principal component analysis for **A:** anchored, **B:** non-anchored and **C:** simultaneous use of both ISSR markers.

Table 5. Principal component analysis of bread wheat cultivars using the similarity matrix.

Components characteristic	Anchored marker			Non anchored marker			Anchored and non anchored marker		
Components No.	1	2	3	1	2	3	1	2	3
Eigen Value	16.21	9.77	7.23	13.87	8.20	5.77	5.95	4.28	3.48
Percentage of variation explained by the first 3 axes	35.44	21.37	15.80	37.24	22.02	15.48	27.69	19.92	16.15
Cumulative percentage of variation explained by the first 3 axes	35.44	56.81	72.61	37.24	59.26	74.74	27.69	47.61	63.76

repeat (ISSR) markers to determine the genetic diversity of 39 bread wheat accessions, including 33 wheat landraces and 6 wheat cultivars from northwest of Iran. The results indicated a high level of polymorphism in wheat landraces based on these markers in contrast to other markers. The cluster analysis suggested that ISSR markers were efficient tools to estimate intra-specific genetic diversity in wheat and this molecular marker could differentiate the local varieties obtained from different locations. Etminan *et al*. (2016) evaluated genetic variation in 25 durum wheat lines and 18 landraces, using 15 inter-simple sequence repeat (ISSR) and six start codon targeted (SCoT) markers. High levels of polymorphism were observed; 98.70% (ISSR) and 100% (SCoT), which indicated these markers are useful tools for detecting genetic variation in the collection.

CONCLUSION

Based on the findings of the present study, the following conclusions were made:

1. The highest and the lowest polymorphism of anchored ISSR markers were observed in winter wheat population by 84.62 and facultative population by 80.77%; respectively. The highest and the lowest levels of polymorphism of non-anchored ISSR markers were observed in facultative wheat population by 80.77% and in winter population by 69.23%, respectively. The average polymorphism attained for anchored ISSR markers and non-anchored ISSR indicates that anchored ISSR markers are more efficient than non-anchored ISSR markers to identify polymorphism.

2. When anchored and non-anchored ISSR markers were used simultaneously for cluster analysis and grouping, the genetic diversity pattern corresponded with geographical diversity and the cultivars of the same geographical regions were placed in an identical group.

3. Generally, anchored ISSR markers performed better than non-anchored ISSR markers in genotype grouping, though the simultaneous use of both markers yielded better results and could separate the cultivars of cold and moderate regions to some extent.

4. With respect to clustering and analyzing the principle component with anchored and non-anchored ISSR markers, it is concluded that a number of cultivars have less genetic similarity compared to the others.

ACKNOWLEDGMENTS

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