

Genetic Diversity of Bread Wheat (*Triticum aestivum* L.) Genotypes Using RAPD and ISSR Molecular Markers

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ABSTRACT

The importance of grain cultivation especially wheat is obvious in terms of providing human and animal food and its impact on the economy of human societies. The reduction of genetic diversity in cultivars prevents increasing yields in line with rising demand and consumption. Therefore, it is necessary to improve the compatibility of them and increase their genetic extent. In the current, the genetic diversity of Iranian wheat genotypes was investigated at the DNA level using RAPD and ISSR markers. 17 RAPD primers and 16 ISSR primers generated 86 (86/99= %86.86) and 56 (56/64= %57.5) polymorphic bands respectively. The cluster analysis based on UPGMA and dendrogram plotted using NTSYSpc 2.02 software revealed three main clusters. The highest genetic distance was between CD-89-2 and CD-89-7 genotypes and the minimum genetic distance was between CD-89-2 and CD-89-3 genotypes. Based on Nei's genetic distance matrix, the mean number of effective bands, the Shannon index, and polymorphism content were 1.381, 0.332, and % 87.12, respectively. Our results showed that RAPD and ISSR analysis are suitable methods to study genetic diversity and relationships among *T. aestivum* genotypes.

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Introduction

Bread wheat (*Triticum aestivum* L.) is one of the oldest and the most important crop with a special strategic value in the World. It is the largest crop in both cultivation and consumption which the increasing population growth has also increased the necessity of its production (FAO, 2006). Besides, changing lifestyles has posed challenges to the wheat breeders to develop newer wheat varieties with high yielding performance, high-quality seed, and resistance to pests and stress conditions (Rana *et al.*, 2013). Like other plant species, wheat genetic diversity is also reducing which causes a reduction in plant resistance following by its various traits defeats through the passage of biological phenomena and natural selection (Allard, 1996). Therefore, assessment of the extent and nature of

genetic variation in bread wheat is important for breeding and genetic resource conservation programs. The study of genetic diversity of plants determines their potential for improved efficiency and hence their use for breeding, which eventually may result in enhanced food production (Khodadadi *et al.*, 2011). A high genetic diversity means that there are more chances of selecting and evolving better varieties. Genetic diversity can be estimated using pedigree analysis, morphological, physiological, and cytogenetic characters or molecular markers (Habash *et al.*, 2009). Molecular markers increase the efficiency of genotypes selection because their expression is independent of environmental effects, more convenient and reliable, and need-less time (Shirmohammadi *et al.*, 2018). As molecular

markers have been developed, they have been extensively explored for analysis of genetic diversity in common wheat. Molecular markers such as restriction fragment length polymorphisms/ RFLP (Barbosa *et al.*, 1996) and randomly amplified polymorphic DNA/ RAPD (Das and Misra, 2010), amplified fragment length polymorphisms/ AFLP (Roy *et al.*, 2004), inter simple sequence repeats/ ISSR (Zietkiewicz *et al.*, 1994) and simple-sequence repeats or microsatellite/ SSR have been used extensively in studies of wheat genetic diversity (Barakat *et al.*, 2011; Ijaz and Khan, 2009). Among these molecular markers, RAPD is a dominant marker that its uses are common due to its simplicity, efficiency, and non-requirement of sequence information. RAPD provides a virtually limitless set of descriptors to compare individual plants and populations. Genetic diversity estimation and screening of genetic resources held in gene banks, natural populations, ecosystems, and natural reserves can be carried out using RAPD as an innovative and fast tool (Tahir, 2008). RAPD analysis has been extensively used for cultivar identification (Malik *et al.*, 1996), fingerprinting genomes (Welsh and McClelland, 1990) and genetic variation studies in *Triticum* (Bedo *et al.*, 2000; Cao *et al.*, 2002; Gupta *et al.*, 2000), *Ziziphora tenuior* (Hatari *et al.*, 2013), *Fritillaria imperialis* (Koochgard *et al.*, 2012), *Cicer arietinum* (Fazeli *et al.*, 2011), *Oryza sativa* (Aghazadeh *et al.*, 2003), *Thymus migricus* (Yavari *et al.*, 2012), *Vitis vinifera* (Jedari *et al.*, 2011), *Asparagus officinalis* (Sarabi *et al.*, 2010).

Due to high annealing temperature and extended sequences like RAPD markers, ISSR primers can produce more reproducible and reliable band patterns. Inter-simple sequence repeat markers are employed for distinguishing DNA based on single base variation or insertions and deletions. For its advantages of a simple procedure, low-cost, good stability, and high reproducibility, ISSR markers have been successfully used in the genetic mapping (Casaoli *et al.*, 2001; Cekic *et al.*, 2001; Tanyolac, 2003). Najaphy *et al.* (2012) revealed that ISSR markers provided sufficient polymorphism and reproducible fingerprinting profiles for evaluation of the genetic diversity of wheat genotypes. ISSR markers are increasingly applied in plant sciences and have detected a

sufficient degree of polymorphism in *Piper nigrum* (Johanson *et al.*, 2005), *Viciifolia onobrychis* (Prevost *et al.*, 1987), *Faba bean* (Terzopoulos and Bebeli, 2008), safflower (Golkar *et al.*, 2011), rice (Blair *et al.*, 1999; Varshney *et al.*, 2005), *Malus orientalis* (Khodadost *et al.*, 2016) and species of the genus *Diploaxis* (Martin and Sanchez-Yelamo, 2000). Thus, the main objective of this study was to estimate the genetic diversity of eight Iranian wheat genotypes using ISSR and RAPD markers.

Materials and Methods

Plant materials

The eight Iranian wheat genotypes from the Research Center for Medicinal Plants, Ministry of Agriculture were grown in sterile Petri dishes in the greenhouse providing normal growing conditions. After about 10-14 days of seed germination, the number of 4-5 seedlings were cut and packed in plastic bags and stored at – 80°C for DNA extraction. A list of these cultivars is presented in table 1.

Table 1. List and code of eight wheat genotypes used in this study.

Name	Genotype code
CD-89-1	1
CD-89-2	2
CD-89-3	3
CD-89-6	4
CD-89-7	5
CD-89-15	6
CD-89-16	7
CD-89-17	8

DNA extraction and PCR conditions

Genomic DNA was extracted from 10-14 days-old young wheat leaves containing the lowest amount of secondary metabolites, using the CTAB method (Doyle and Doyle, 1987). The PCR reaction mixture for both RAPD and ISSR amplification assay had a total volume of 15 µl, which contained 1.5 ng genomic DNA, 7.5 µl DFS Master mix (BioRon, Germany; containing 1 unit *Taq* DNA polymerase, 0.1 mM of each dNTPs, 2.5 mM MgCl₂, 0.01% Tween 20, 65 mM Tris- HCl and 16 mM (NH₄)₂SO₄) and 6 µl (60 pmol) primer. Seventeen RAPD primers and sixteen ISSR primers were used for PCR reaction (Tables 3 and 4). The amplification protocol for each technique is presented in table 2.

Table 2. The amplification program of RAPD and ISSR.

Cycles	Step name	Temperature / Time for RAPD	Temperature / Time for ISSR	Aim
40-45	Initialization	94 °c / 4 min	94 °c / 5 min	Initial being single strand
	Denaturation	94 °c / 45 sec.	94 °c / 1 min	General being a single strand
	Annealing	27 & 30 °c / 1 min	35-59 °c / 1 min	Primer Connection
	Extension	72 °c / 1-2 min	72 °c / 1 min	Synthesis DNA
	Final elongation	72 °c / 7 min	72 °c / 10 min	Total DNA synthesis
	Final hold	4 °c / ∞	4 °c / ∞	Final hold

The amplified products were separated on 2 % agarose gels and stained with DNA Safe Stain (Cina Gen, Iran). Images were photographed, captured by Gel Doc 1000 Gerix (Biostep Co., Germany).

Data analysis

Amplified products were scored as band presence (1) or absence (0) and binary matrices were assembled for the markers. The genetic similarity was calculated with the unweighted pair group method using arithmetic average (UPGMA) as a clustering algorithm. The dendrogram was drawn using SAHN and cluster analysis performed via a complete linkage method using NTSYS-pc software version 2.02 (Rolf, 1998). Then, Nei distance matrices and genetic similarity were drawn using the GenALex 6.503 software, and the percentage of polymorphism was calculated.

Results

All genotypes studied were screened and amplified by 17 RAPD and 16 ISSR primers. All of the primers produced reproducible and scorable amplification products. Tables of 3 and 4 show codes and sequences of primers, T_m (melting temperature), T_a (annealing temperature), the number of polymorphic and total bands resultant from all tested wheat genotypes. Electrophoresis of RAPD products showed 99 bands on agarose gel that its 86 bands were polymorphic and 13 bands were monomorphic. Molecular sizes of amplified fragments ranged from 150 to 1500 bp. The number of DNA fragments for each primer varied from 1 (RP18) to 9 (RP13 and RP15) with average fragments of 5.82 and the average polymorphic fragments per primer was determined as 5.05.

Table 3. List of RAPD primers along with their sequences, T_m , T_a , the number of polymorphic and total bands resultant from all tested wheat genotypes.

Primer	Sequence (5'→3')	T_m	T_a	Total bands	Polymorphic bands	Polymorphismratio (%)
RP 1	TGCCCCGTCGT	34	30	3	3	100
RP 2	ACAACGCCTC	32	27	4	4	100
RP 3	TGCCGAGCTG	34	30	8	8	100
RP 4	GGGTAACGCC	34	30	6	5	83.3
RP 5	GGTGAACGCT	32	27	4	3	75
RP 6	GGACCCAACC	34	30	3	3	100
RP 7	TGCGCCCTTC	34	30	7	5	71.4
RP 11	TCGGCGATAG	32	27	6	6	100
RP 12	GGGAATTCGG	32	27	7	6	85.7
RP 13	CCAAGCTTCC	32	27	9	8	88.8
RP 14	CCGATATCCC	32	27	8	7	87.5
RP 15	GTAGCACTCC	32	27	9	7	77.7
RP 16	TCTGTGCTGG	32	27	8	7	87.5
RP 17	GACGGATCAG	32	27	2	2	100
RP 18	GTGACGTAGG	32	27	1	1	100
RP 19	AAGAGCCCGT	32	27	6	5	83.3
RP 20	TTCAGGGTGG	32	27	8	6	75
Total mean				99	86	86.86

Table 4. List of ISSR primers along with their sequences, T_m , T_a , the number of polymorphic and total bands resultant from all tested wheat genotypes.

primer	Sequence (5'→3')	T_m	T_a	Total bands	Polymorphic bands	Polymorphism ratio (%)
IP 1	GAGAGAGAGAGAGACT	48	45	2	2	100
IP 2	AGAGAGAGAGAGAGCA	54	50	3	3	100
IP 3	GTGTGTGTGTGTGTGTC	52	47	1	1	100
IP 4	CTCTCTCTCTCTCTA	50	45	7	6	85.7
IP 5	ACACACACACACACACT	56	51	7	6	85.7
IP 6	ACACACACACACACACG	58	53	5	2	40
IP 7	TCTCTCTCTCTCTCTCG	52	47	7	6	85.7
IP 8	ATGATGATGATGATGATG	48	45	2	2	100
IP 9	GAAGAAGAAGAAGAAGAA	48	45	2	2	100
IP 10	ACGTGTGTGTGTGTGTG	52	47	2	2	100
IP 11	AGAGAGAGAGAGAGAGTC	54	50	5	5	100
IP 12	GAAGAAGAAGAAGAA	40	35	4	4	100
IP 13	ACCACCACCACCACCACCG	64	59	5	5	100
IP 14	ACACACACACACACACCG	56	51	4	4	100
IP 15	CAGCACACACACACACA	58	53	5	3	60
IP 16	AGAGAGAGAGAGAGAGCG	56	51	3	3	100
Total mean				64	56	87.5

The ratio of polymorphic bands number / the total number of amplified fragments ranged between 71.4 % (PR7) and 100 %, with an average of 86.86 % (86.86 % polymorphism). A total of 64 bands were obtained from ISSR products which 56 were polymorphic and 8 were monomorphic across the wheat genotypes studied. The fragment size ranged from 150 - 550 bp, but there were rarely 700 and 800 base pairs. The IP3 primer with one band had the least number of bands and IP4, IP5 and IP7 primers with seven bands had the highest number of bands. An average fragment was four and

polymorphic fragments per primer were determined an average of 3.5. The ratio of the number of polymorphic fragments / the total number of amplified fragments ranged between 40 % (IP6) and 100 %, with an average of 87.5 %. The genetic similarity among the varieties ranged from 64.4 % to 85.27 %. Maximum genetic similarity was observed between CD-89-2 and CD-89-3 genotypes, closely followed by a comparison between CD-89-1 and CD-89-3 or CD-89-6 and CD-89-7 (GS= 84.66 %). On the contrary, the lowest genetic similarity was between CD-89-2 and CD-89-7 (Table 5).

Table 5. The similarity matrix of eight wheat genotypes.

Genotype	CD-89-1	CD-89-2	CD-89-3	CD-89-6	CD-89-7	CD-89-15	CD-89-16	CD-89-17
CD-89-1	1.0000000							
CD-89-2	0.7852761	1.0000000						
CD-89-3	0.8466258	0.8527607	1.0000000					
CD-89-6	0.7423313	0.6748466	0.6993865	1.0000000				
CD-89-7	0.6871166	0.6441718	0.6687117	0.8466258	1.0000000			
CD-89-15	0.6871166	0.6564417	0.6464417	0.7607362	0.7300613	1.0000000		
CD-89-16	0.7116564	0.6809816	0.7423313	0.7239264	0.7423313	0.7177914	1.0000000	
CD-89-17	0.6993865	0.7177914	0.7423313	0.6503067	0.6687117	0.6564417	0.7791411	1.0000000

According to the obtained results, the cultivars can be divided into three distinct groups stating that these three groups of genotypes have been derived in the past (Fig. 1). Then, we were able to calculate the Nei genetic matching and genetic

distance matrices for these three groups using the GenALex 6.503 software (Table 6). The average number of effective bands was 1.381, the Shannon index was 0.332, and polymorphism was 87.12%, using GenALex 6.503 software.

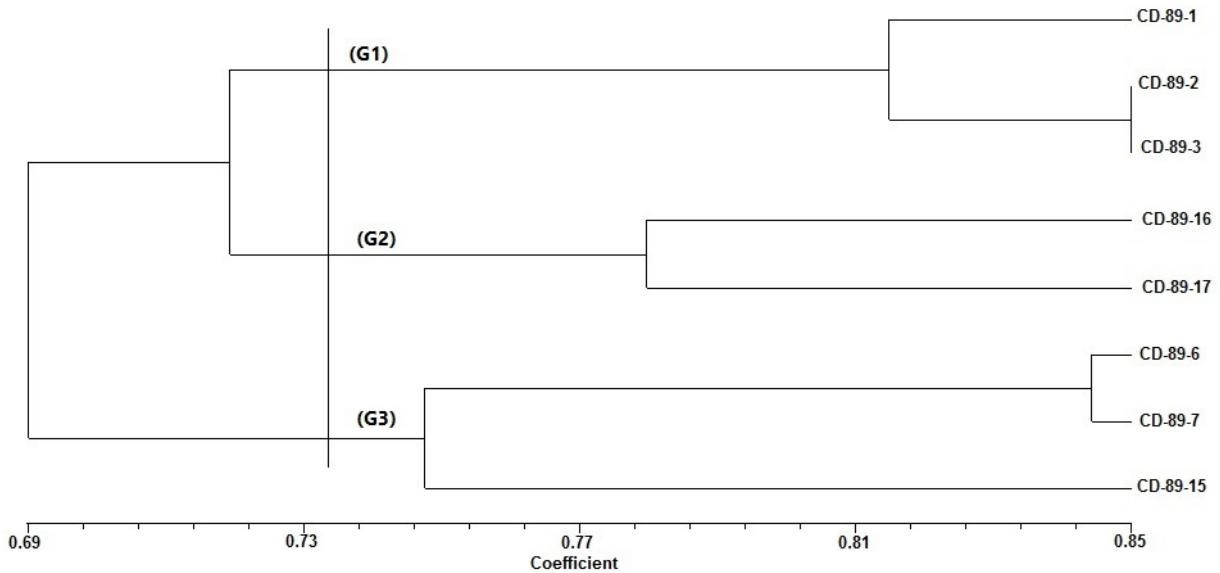


Fig. 1. UPGMA dendrogram showing the relationships among eight wheat genotypes.

Table 6. Nei's genetic distance and similarity matrix for three wheat groups.

Genotypes	Nei's genetic distance matrix			Nei's genetic similarity matrix			
	Groups	G1	G2	G3	G1	G2	G3
CD-89-1	G1	0			1		
CD-89-2							
CD-89-3							
CD-89-16	G2	0.288	0		0.75	1	
CD-89-17							
CD-89-6	G3	0.176	0.214	0	0.839	0.807	1
CD-89-7							
CD-89-15							

Discussion

According to the obtained results, CD-89-1, CD-89-2, and CD-89-3 could be grouped into a group and expressing that these three digits were more closely interrelated in the past. Although, this state can apply to the CD-89-16 and CD-89-17 or even CD-89-6 and CD-89-7 varieties. But the derivation of CD-89-15 from these two cultivars (CD-89-6 and CD-89-7) goes back a long time. The highest genetic distance was between CD-89-2 and CD-89-7 and the minimum genetic distance was between CD-89-2 and CD-89-3. Genetic diversity studies in plant species using molecular markers such as RAPD, ISSR, AFLP, RFLP, etc, have been successfully used by very researchers. Among them, RAPD and ISSR markers have been successfully used in wheat genetic diversity evaluation, because of their many advantages (Sofalian *et al.*, 2008; Abdellatif *et al.*, 2011; Cifci and Yagdi, 2011; Kumar *et al.*, 2017; Eid, 2018).

Both RAPD and ISSR Markers are quick and easy to handle and have been successfully utilized for the phylogenetic analysis (Patra, 2011). RAPD gained importance due to its simplicity, efficiency, and non-requirement of sequence information. These markers provide a virtually limitless set of descriptors to compare individual plants and populations (Cifci and Yagdi, 2011). ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping, and evolutionary biology (Siddiq *et al.*, 2002).

In this study, we used the RAPD and ISSR genetic markers for the study of genetic diversity in eight Iranian bread wheat cultivars. Of the two molecular marker systems used, 20 RAPD primers produced a total of 99 DNA fragments, whereas 16 ISSR primers produced only 64 DNA fragments. It has been reported that the ability to decision genetic variation may be more directly related to the degree of polymorphism

detected by the marker system (Sivaprakash *et al.*, 2004). In this work, the level of polymorphism revealed by RAPD (86.86%) was almost equal to ISSR (87.5%). In agreement with our results, Sofalian *et al.*, (2008) detected 82.20% of polymorphism in 39 bread wheat landraces and cultivars using 9 ISSR primers; Abdellatif *et al.*, (2011) detected 69.15 % of polymorphism in 14 bread wheat cultivars using 17 RAPD primers; Eid (2018) detected 73% of polymorphism in 5 wheat genotypes using RAPD markers. Also, similar results reported about other crops like rice been by Muthusami *et al.*, (2008), 70.30% of polymorphism by RAPD and 60.79% by ISSR, and by Yu and Nguyen (1994), 80% of polymorphism in 13 *Oryza sativa* cultivars with RAPD markers.

However, the results of the quantitative and qualitative comparison of the fatty acids and proteins of these eight cultivars studied, which were carried out by Arekesh (2014), have produced different dendrograms. In that study, the most similarity between CD-89-1 and CD-89-7 genotypes, and the greatest difference was between CD-89-3 and CD-89-6 genotypes (Arekesh, 2014). This difference can be justified by the fact that the primers used in this study may not be studied for the sequences of genes related to fatty acids and proteins of eight wheat cultivars due to being random sequences of 10 nucleotides of the genome and being not limited to a specific gene. The results of protein measurement with a specific molecular weight such as 120 kDa, 85 kDa, and 25 kDa by Arekesh (2014) are similar to our research results. Based on our data, the amount of each fatty acids found in the eight wheat cultivars studied showed no significant relation with the results of Arekesh (2014), which have been investigated in six periods. Also, CD-89-15 genotype has the lowest amount of arachidonic, linolenic, EDP, and palmitoleic acids, and the highest stearic acid content which the difference with other genotypes is also evident in our research.

Since genetic distance is one of the most important factors to select parents for breeding programs, cultivars that have been placed in different groups can be identified as potential parents in wheat breeding programs.

Conclusion

The present study was aimed to determine the genetic variation among eight Iranian bread wheat cultivars using RAPD and ISSR markers. Our results showed that the highest genetic distance was between CD-89-2 and CD-89-7 and the minimum genetic distance was between CD-89-2 and CD-89-3. Also, our data could be suggested that genetic variation among the eight Iranian wheat cultivars studied is less than expected, and these eight cultivars are more similar together. However, we intend to conduct further studies using other molecular markers on these wheat cultivars so that we can use the results of all studies to more accurately judge the genetic diversity of these eight cultivars.

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Conflicts of interest

The authors have declared that no competing interests exist.

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