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RESEARCH ARTICLE

Elucidate Genetic Diversity and Population Structure of Bread Wheat (*Triticum Aestivum* L.) Cultivars Using IRAP and REMAP Markers

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Abstract

Analysis of genetic diversity and population structure in bread wheat is an essential step in their conservation, utilization, and breeding. Retrotransposons are ubiquitous and abundant a throughout the plant genomes, therefore extensively used as ideal molecular markers for genetic variability, DNA fingerprinting and genetic mapping studies in plant species. In the current research, we used two retrotransposon-based marker systems, inter-retrotransposon amplified polymorphisms (IRAPs), and the retrotransposon-microsatellite amplified polymorphisms (REMAPs) markers to evaluate the genetic diversity and survey activity of long terminal repeat retrotransposon (LTR-retrotransposon) elements in a collection of 49 bread wheat (*Triticum aestivum* L.) cultivars that mainly bred in Iran. In general, 90 and 126 loci were amplified using 9 IRAP and 20 REMAP primers, respectively. Both techniques produced a satisfactory number of bands for cultivar analysis; however, the technique IRAP, particularly single primer Nikita generated a large number of bands, indicating the wide activity of Nikita family under various environmental conditions of bread wheat. The percentage of polymorphic loci (PPL) in the studied collection for IRAP and REMAP markers was 81.78 and 86.40%, respectively. A model-based Bayesian method, Principal coordinate analysis (PCoA) and cluster analysis using Minimum Evolution (ME) algorithm hinted of the existence of two groups. This grouping was in agreement with the growing season and conformed by the high within-group bootstrap value. These results demonstrated that these markers developed using transpositionally active retrotransposons (RTNs) are efficient and reliable markers in determining level of genetic diversity and population structure in bread wheat in breeding programs.

Key words : Genetic diversity, model-based cluster, *Triticum aestivum* L., LTR retrotransposon, population structure

Introduction

Bread wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD) is one of the foremost stable food crops in the human diet worldwide and widely grown crops in many countries, including in Iran. In the cradle of agriculture, Iran is considered as a primary center of wheat genetic diversity and a recent study indicated Caspian Iran to be the main source of the wheat D genome (Salamini et al. 2002; Wang et al. 2013) therefore Iranian wheat landraces are precious genetic resources for new alleles or genes to be used in breeding for new cultivars and the survival of future generations (Ciaffi et al. 1992).

Genetic drift, reduced gene flow, possible local selection, domestication processes, and recently the recurrent use of adapted germplasm, modern agricultural methods, and continuous breeding practices have drastically compromised the genetic diversity of major crops such as bread wheat (Aremu

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et al. 2007; Donini et al. 2000; Nasri et al. 2013). Roussel et al. (2004, 2005) demonstrated a decline in allelic diversity after the 1960s in a collection of 559 French bread wheat accessions from the years 1800-2000 using 42 simple sequence repeats (SSRs), and in 480 European wheat cultivars from 1840-2000 using 39 SSRs. A thorough knowledge of the population structure and genetic diversity spectrum of protected plant species are a prerequisite for their protection, management, and plant genetic resource utilization. However, genetic variability plays a major role in dealing with different biotic and abiotic stresses (Kumar and Agrawal 2017), and the accurate estimation of genetic variation in a germplasm is crucial for the survival, evolution, effective conservation, and more efficient utilization of genetic resources in crop improvement programs (Kabbaj et al. 2017; Laurentin 2009). Therefore, the assessment of genetic diversity in bread wheat germplasm provides fundamental and useful information and broadens the genetic variation in future breeding programs (Uddin and Boerner 2008). Traditionally, genetic variation analyses relied on morphological and phenotypic markers (Liu et al. 2016), but these markers have been restricted to a few phenotypic traits strongly affected by environmental conditions and exhibit little variation, especially for highly heritable traits, therefore, the use of morphological and phenotypic markers might have some limitations and instability (Rao 2004). In contrast, DNA-based molecular markers have become the most effective tool and fast method for assessment of genetic diversity and structure in a plant collection in recent years (Abouzied et al. 2013) because they can overcome many of the limitations associated with phenotypic-based diversity analysis, are plentiful, and allow cultivar identification at early stages of plant development (Manifesto et al. 2001).

With characteristics of dispersion, ubiquity, prevalence, high heterogeneity, and genomic dynamism of retrotransposon-like elements in plant genomes can be exploited for DNA-fingerprinting using Inter-Retrotransposon Amplified Polymorphism (IRAP) and Retrotransposon-Microsatellite Amplified Polymorphism (REMAP) markers (Kalendar et al. 2011; Schulman et al. 2012). The IRAP method displays insertional polymorphisms by amplifying the DNA segments between two nearby retrotransposons (RTNs) using outward-facing primers, whereas REMAP produces products between a long terminal repeat (LTR) primer and a microsatellite motif. Retrotransposons contain long, nested, defined, conserved sequences and are dynamic in their induction both by biotic and abiotic stresses (Galindo-Gonzalez et al. 2017; Grandbastien et al. 2005; Jaaskelainen et al. 2013; Vuorinen et al. 2018). The advantage of the application of these marker systems consists in the genomic localization, structure and replication strategy, high integration activity, capability for tracking an insertion event, and its subsequent vertical radiation through a pedigree or phylogeny (Kalendar et al. 2011; Shimamura et al. 1997). For these properties, retrotransposons have become the markers of choice for genetic diversity studies in many plant species in recent decades. Genetic diversity of Iranian bread wheat has been evaluated in the past by using different molecular markers such as SSR (Mohammadi et al. 2009; Zarei Abbasabbad et al. 2016), IRAP and REMAP (Nasri et al. 2013).

In the present study, we developed and used IRAP and REMAP markers to find useful retrotransposon markers for diversity analyses, genetic structure, and to assess the polymorphism of these markers among spring, winter, and facultative cultivars of bread wheat with the aim of using them in breeding programs as well as for conservation management of this germplasm. Since the retroelements are active under the influence abiotic stress, such as drought, salinity, cold, heat, these stress factors induce mutations that could help the organism adapt to new environmental conditions, therefore another aim of this study is survey activity of retroelements in 49 bread wheat, which all differ in their stress background.

Materials and Methods

Plant material and DNA isolation

Plant material consisted of 49 Iranian bread wheat (*Triticum aestivum* L.) cultivars mostly used in breeding for biotic and abiotic stress, kindly provided by the University of Tabriz (Prof Seyed Abolghasem Mohammadi), and Seed and Plant Improvement Institute, Karaj, Iran (Supplementary Table 1). Seeds were planted in small pots with 10 cm diameter containing a mixture of garden soil and vermiculite in the greenhouse with an ambient of temperature 25°C. Genomic DNA was extracted from young leaves of 25-day-old seedlings using the method described by Ausubel et al. (1995) with minor modifications. The quantity and quality of DNA were assessed using Biophotometer (Eppendorf, Germany) and 1% (w/v) agarose gel electrophoresis, respectively.

IRAP and REMAP reactions

Six single and 15 IRAP primer combinations (Tables 1 and 2) were used to analyze genetic diversity and integration events of retrotransposons in 49 bread wheat (*Triticum aestivum* L.) cultivars. The IRAP and REMAP PCR amplifications were carried out in a Bio-Rad thermo cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total volume of 25 µl containing 12.5 µl Master mix (Taq DNA polymerase, 10 × PCR buffer, dNTPs, MgCl2), 1 µl primer, 8.5 µl ddH20 and, 3 µl of genomic DNA template. The amplification profile was composed of an initial denaturation at 94°C for 3 min, followed by 45 cycles of 94°C for 60 s, annealing at each primer combination temperature (Table 2) for 60 s, and 72°C for 2 min, with a final extension of 7 min at 72°C. The PCR products were resolved by electrophoresis (Bio-Rad) using 1.5% Resolute TM line Biozyme agarose gel in 0.5 × TBE buffer with constant voltage of 70 V for 3-4 h. Gels were stained by ethidium bromide, then DNA fragments were visualized under UV light and photographed using a gel

Table 1. Primer name, retrotransposon type, position, and sequences.

Name and orientation	Origin in barley	Position	Sequence
Nikita →	Nikita	1-22	CGCATTTGTTCAAGCCTAAACC
Sukkula →	Sukkula	4301-4326	GATAGGGTCGCATCTTGGGCGTGAC
LTR6149 →	BARE-1	1993-2012	CTCGCTCGCCCACTACATCAACCGCGTTTATT
LTR6150 ←	BARE-1	418-439	CTGGTTCGGCCCATGTCTATGTATCCACACATGTA
5′LT R1 ←	BARE-1	1-26	TTGCCTCTAGGGCATATTTCCAACA
5′LT R2 ←	BARE-1	314-338	ATCATTCCCTCTAGGGCATAATTC
UBC808 (ISSR)			$(AG)_8C$
UBC812 (ISSR)			$(GA)_8A$
UBC835 (ISSR)			$(CAC)_7G$
UBC837 (ISSR)			$(CA)_{10}G$
UBC838 (ISSR)			$(GTG)_7C$
UBC864 (ISSR)			$(CAC)_7T$
UBC865 (ISSR)			$(CAC)_7GT$

Table 2. Characteristics of the IRAP and REMAP primers used in this study.

Primer name	Tm	TL	PL	PPL	Ne	He	I	Band size (bp)
IRAP								
Nikita	55	14	13	93	1.78	0.42	0.60	75-3000
Sukkula	56	13	11	85	1.22	0.16	0.28	100-2500
LTR6150	56	11	9	82	1.75	0.39	0.55	300-2000
LTR6149	55	9	8	88	1.71	0.39	0.56	75-2000
Nikita/LTR6150	57	7	6	86	1.62	0.35	0.51	300-3000
Nikita/LTR6149	58	5	4	80	1.65	0.36	0.51	75-1000
Sukkula/Nikita	55	9	6	67	1.31	0.20	0.32	100-1500
Sukkula/ LTR6150	57	10	8	80	1.23	0.18	0.30	100-2000
Sukkula/ LTR6149	57	12	9	75	1.25	0.18	0.30	100-2000
Total		90	74					
Average		10	8.22	81.78	1.50	0.29	0.44	
REMAP								
Nikita/UBC808	56	10	9	90	1.63	0.35	0.52	75-2500
Nikita/UBC835	55	8	8	100	1.90	0.47	0.67	75-2000
Nikita/UBC865	58	3	3	100	1.95	0.49	0.68	200-1500
Nikita/UBC838	57	4	4	100	1.90	0.47	0.67	300-1500
Nikita/UBC812	56	6	6	100	1.79	0.44	0.63	300-2000
Sukkula/UBC808	55	8	6	75	1.40	0.26	0.39	100-2500
Sukkula/UBC812	55	7	4	57	1.38	0.22	0.33	100-2000
Sukkula/UBC835	57	9	7	78	1.50	0.30	0.44	100-2500
Sukkula/UBC838	56	6	4	67	1.44	0.26	0.38	100-2000
Sukkula/UBC864	57	8	5	62	1.50	0.27	0.40	75-2000
Sukkula/UBC865	58	9	6	67	1.49	0.28	0.41	100-3000
LTR6150/UBC812	55	6	6	100	1.70	0.40	0.58	200-2000
LTR6150/UBC835	57	6	4	66	1.53	0.29	0.42	75-1500
LTR6150/UBC838	58	8	8	100	1.67	0.40	0.58	300-3000
LTR6150/UBC837	57	3	3	100	1.33	0.24	0.40	100-1500
LTR6150/UBC864	55	6	5	83	1.76	0.39	0.56	100-2000
LTR6150/UBC865	57	4	4	100	1.88	0.47	0.66	300-1500
LTR6149/UBC835	58	4	4	100	1.70	0.39	0.58	100-1500
LTR6149/UBC864	58	5	5	100	1.82	0.45	0.64	400-2000
5′LTR1/UBC865	56	6	5	83	1.72	0.38	0.54	200-1500
Total		126	106					
Average		6.30	5.30	86.40	1.65	0.36	0.52	

TM:Annealing temperature, *TL*: total loci, *PL*: polymorphic loci, *PPL*: percentage of polymorphic loci, *He*: expected heterozygosity, *Ne*: number of effective alleles, *I*: Shannon's information index.

documentation system.

Thirty-five REMAP primer combinations, derived from five single IRAP primers with seven Inter-Simple Sequence Repeats (ISSR) primers (Tables1 and 2) were applied. First, all IRAP and REMAP primers were tested on three bread wheat cultivars genotypes to choose the primers producing scorable and discernible banding patterns.

Data analysis

The amplified fragments were scored independently for their presence (1) or absence (0) at each position. Weak bands were not scored. The number of bands, percentage of polymorphic bands (PPB), mean of expect heterozygosity (He), standard error of mean heterozigosity, number of effective alleles (Ne), and Shannon's information index (I) were calculated. Furthermore, genetic differentiation levels between winter, facultative, and spring groups were calculated with GenAlEx 6.4 (Peakall and Smouse 2006) software based on IRAP, REMAP, and combined IRAP/REMAP data. To check the goodness fit of a cluster analysis, the cophenetic correlation coefficient was calculated to evaluate the adjustment between similarity matrices and respective dendrogram-derived matrices (cophenetic matrix). To estimate the degree of correlation among the three cophenetic matrices derived from IRAP, REMAP, and combined data, Mantel test was performed with NTSYSpc (Rohlf 2000). A cluster analysis was performed to generate dendrograms using Minimum Evolution (ME) algorithm and number of differences evolutionary distance coefficient in MEGA 4.0 (Tamura et al. 2007). The statistical stability of the cluster was also estimated by a bootstrap analysis using this software. In addition, model-based clustering implemented in Structure 2.3.1 software (Pritchard et al. 2000) was used for the population structure. The analysis was performed based on no admixture model with 50,000 generations of burn in period, 1,000,000 Markov Chain Monte Carlo (MCMC) iterations. The number of sub-populations (*K*) was from 1 to 10 and the most likely *K* value was determined by the log likelihood of the data [LnP(D)] and an *ad hoc statistic, ΔK*, in the web-based software STRUCTURE HARVESTER version 0.6.92 (Earl and Von Holdt 2012). In order to assign the accessions reliably to a given cluster, the estimated membership coefficient of cultivars were measured using this software as well. Principal coordinate analysis (PCoA) was performed using GenAlEx 6.4 (Peakall and Smouse 2006).

Results

Polymorphism of IRAP markers

Out of nine IRAP primers tested (Table 2), four single primers (Nikita, Sukkula, LTR6150, LTR6149) and five primer combinations (Nikita/LTR6150, Nikita/LTR6149, Sukkula/Nikita, Sukkula/LTR6150, Sukkula/LTR6149) produced 90 distinguishable and scorable loci, out of which 74 loci (81.78%) were polymorphic. Length of the amplified fragments ranged from 75 to 3,000 bp. Single primer Nikita generated the maximum amplified, polymorphic loci, percentage of polymorphic loci, and showed the highest Ne, He, and I (Table 2). The lowest values of Ne, He, and I parameters were achieved for the single primer Sukkula. The average of polymorphic loci was 8.22 per primer. Fig. 1 shows the Nikita-based insertion profiles of 20 bread wheat on 1.5% agarose gels.

To assess the genetic relationships of bread wheat cultivars, a phylogenetic tree was constructed using the Minimum Evolution Algorithm and number of difference evolutionary distance coefficient based on IRAP data (Fig. 2). Bootstrap analysis confirmed the reliability of the grouping. High within-group bootstrap values with some exceptions were associated with reduced within-group values compared with between-group genetic diversity. This grouping well resembled the subdivisions that were in agreement with available pedigree information. In the resulting tree, the cultivars were divided into two main groups, and these groups were then

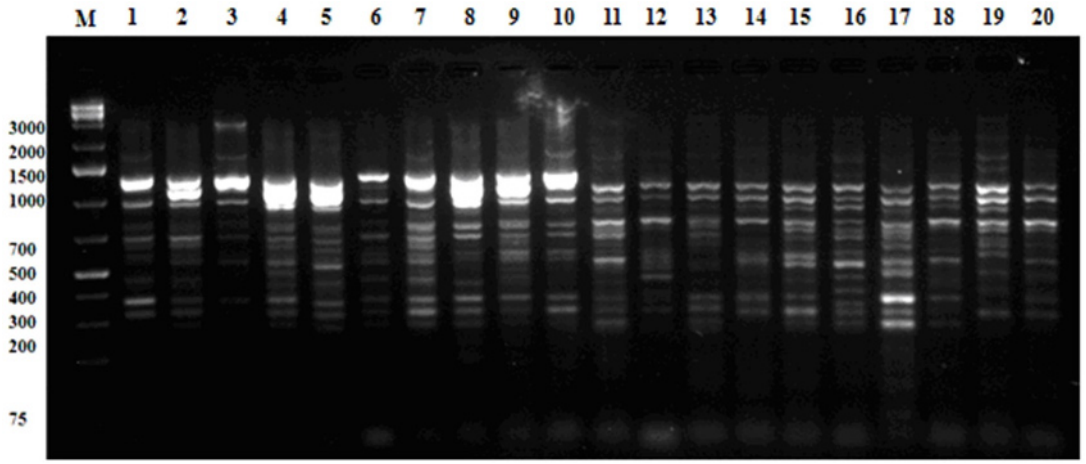


Fig. 1. Polymorphism detected by IRAP primer Nikita. Lane M: 1kb O'GeneRuler^{TM} DNA ladder (Fermentas) in base pair, Lanes 1 to 20 correspond to bread wheat cultivars: Zagros, Arg, Mahdavi, Bam, Arta, Bolani, Mehrgan, Sirvan, Roshan, Baharn, Moghan3, Narin, Alvand, Azar2, MV17, Gaspard, Urom, Karj2, Saison, Navid.

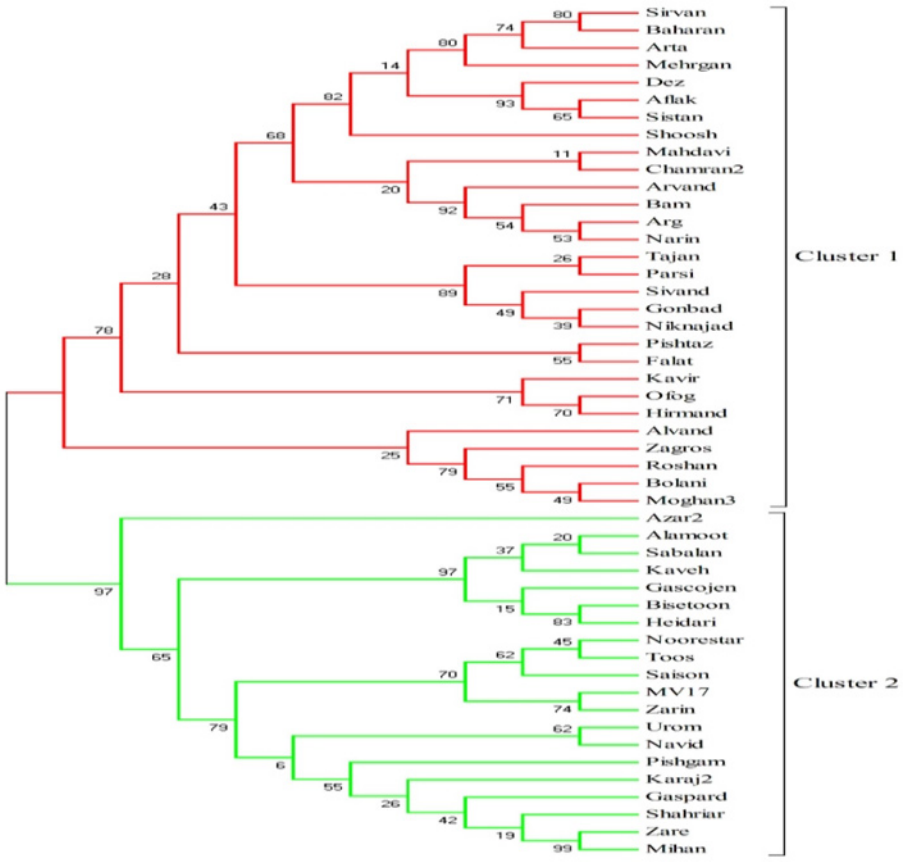


Fig. 2. Grouping of 49 bread wheat cultivars based on IRAP data using Minimum Evolution clustering algorithm and number of difference evolutionary distance coefficient.

separated into several subgroups. Cluster I consisted of all spring cultivars and three facultative cultivars, namely Mahdavi, Bam, and Alvand. In this grouping, six cultivars, Sirvan, Baharan, Arta, Aflak, Sistan, and Dez originating from the CIMMYT were closely clustered together. Kavir, Ofog, and Hirman, three salt-tolerant genotypes, were also grouped together with very high bootstrap value. All the winter and the rest of facultative cultivars were assigned into cluster II. These cultivars were grouped together with bootstrap value of 97, indicating that grouping of the cultivars together in 97% of the case. In this grouping, most the exotic cultivars (Saison, Noorestar, Zarin, MV17, and Toos) were grouped together. Pedigree information available showed that the grouping is associated with the entries pedigree and relatedness.

Polymorphism of REMAP markers

Twenty combinations generated using five RTN and seven ISSR primers combinations amplified 126 fragments in the 49 bread wheat cultivars with 86% polymorphism (106 fragments) and size of 75 to 3000 bp. All RTN primers, except 5′LTR2in combination with at least one ISSR primer generated distinguishable and polymorphic banding patterns. The average of REMAP polymorphic fragments per primer combination was 5.30 and Nikita/UBC808 combination produced the highest number of bands (10) and polymorphic bands (9). The banding patterns generated by LTR6150/UBC812, Nikita/UBC838, Nikita/UBC812, Nikita/UBC835, LTR6150/UBC838, LTR6150/UBC837, LTR6150/UBC865, Nikita/UBC865, LTR6149/UBC835, and LTR6149/UBC864 primer combinations were 100% polymorphic. The mean Ne, I, and He values were 1.65, 0.52, 0.36, respectively, and the highest values of these parameters were recorded for Nikita/UBC865 primer combination (Table 2).

Minimum evolution dendrogram using REMAP markers clearly split 49 cultivars into two groups (Fig. 3). The REMAP-based tree clearly indicates that all of spring cultivars with three facultative cultivars namely Mahdavi, Bam, and Alvand formed a well-consolidated cluster I. Winter cultivars and the rest of facultative cultivars fell into cluster II.

Combined data analysis

To compare the efficiency of IRAP and REMAP markers, different diversity parameters Ne, I, He, and PPL were separately calculated for spring, winter, facultative, and total cultivars. At the cultivar level, facultative cultivars showed the highest value for all diversity parameters by REMAP data (I=0.48, He=0.33, Ne=1.59, PPL=%84.13, respectively) (Table 3). The lowest value for these parameters were recorded for winter cultivars for IRAP markers (I=0.26, He=0.17, Ne=1.28, PPL=%56.67, respectively).

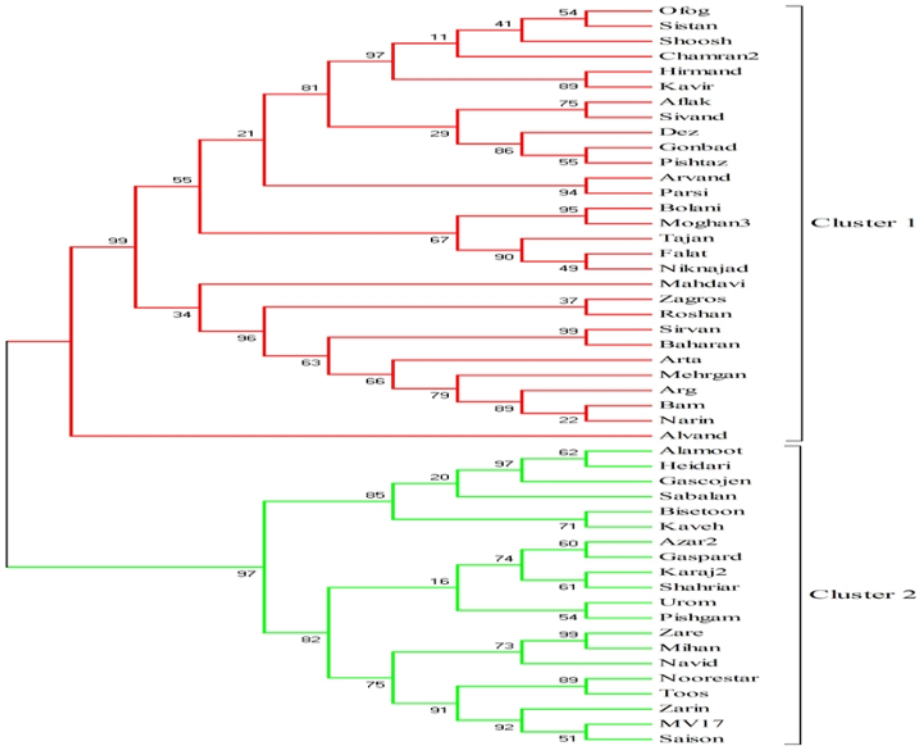


Fig. 3. Grouping of 49 bread wheat cultivars based on REMAP data using Minimum Evolution clustering algorithm and number of difference evolutionary distance coefficient.

Table 3. Diversity parameters estimated based on IRAP, REMAP, and IRAP/REMAP markers in bread wheat cultivars.

Molecular Markers	Cultivar	I (SE)	He (SE)	Ne (SE)	PPL (%)
IRAP	Spring	0.37 (0.028)	0.24 (0.020)	1.41 (0.039)	72.22
	Winter	0.26 (0.029)	0.17 (0.020)	1.28 (0.038)	56.67
	Facultative	0.41 (0.027)	0.27 (0.019)	1.47 (0.038)	82.22
	Total	0.35 (0.016)	0.23 (0.012)	1.39 (0.023)	70.37
REMAP	Spring	0.47 (0.022)	0.32 (0.016)	1.57 (0.032)	81.75
	Winter	0.35 (0.025)	0.23 (0.018)	1.40 (0.034)	64.29
	Facultative	0.48 (0.022)	0.33 (0.016)	1.59 (0.032)	84.13
	Total	0.43 (0.014)	0.29 (0.010)	1.52 (0.019)	76.72
IRAP/REMAP	Spring	0.43 (0.018)	0.29 (0.013)	1.50 (0.025)	77.78
	Winter	0.31 (0.019)	0.20 (0.014)	1.35 (0.026)	61.11
	Facultative	0.45 (0.017)	0.31 (0.012)	1.54 (0.025)	83.33
	Total	0.40 (0.011)	0.27 (0.008)	1.46 (0.015)	74.07

I: Shannon's information index, *SE*: Standard error, *He*: Mean of expected heterozygosity, *Ne*: Number of effective alleles, *PPL*: Percentage of polymorphic Loci.

LTR-RTs of the Nilita, Sukkula, LTR6150, LTR6149, and 5′LTR1 families were shown to differ in integration site distribution in the A, B, and D genomes of hexaploid wheat. Primer Nikita generated the percentage of polymorphic loci, and showed the highest Ne, He, and I, whereas the lowest values of Ne, He, and I parameters were achieved for the single primer Sukkula. Sukkula retrotransposon produced the highest total loci and polymorphic loci, and Nikita retrotransposon with 66 and 59 fragments were ranked second. 5′ LTR1 did not amplify any band in the IRAP system, but generated five polymorphic loci in the REMAP system (Table 4).

Cophenetic matrices of IRAP and REMAP markers were significantly correlated with the IRAP/REMAP data, but Mantel test between IRAP and REMAP cophenetic matrices evidenced no significant correlation (r=0.073). Hence, IRAP/

Table 4. Comparison of Retrotransposon family in studied bread wheat.

Retrotransposon Family	TL	PL	PPL	Ne	He	I
Nikita	66	59	90.66	1.73	0.39	0.57
Sukkula	91	66	71.30	1.37	0.23	0.35
LTR6150	61	53	88.55	1.61	0.35	0.51
LTR6149	35	30	88.60	1.63	0.35	0.52
5′LTR1	6	5	83	1.72	0.38	0.54

I: Shannon's information index, *He*: Mean of expected heterozygosity, *Ne*: Number of effective alleles, *PPL*: Percentage of polymorphic Loci. *TL*: total loci, *PL*: polymorphic loci.

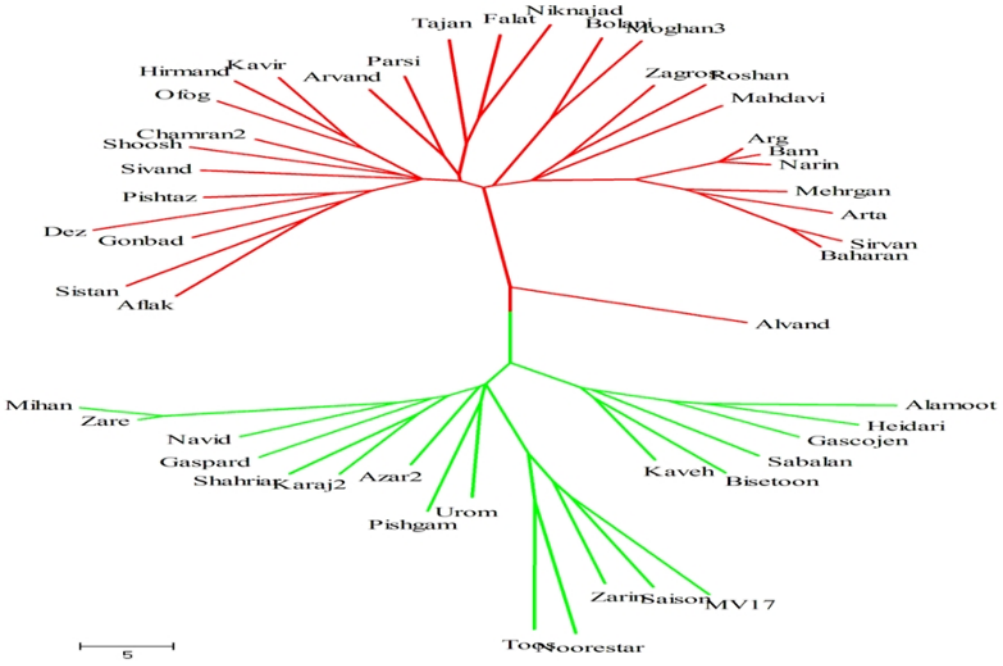
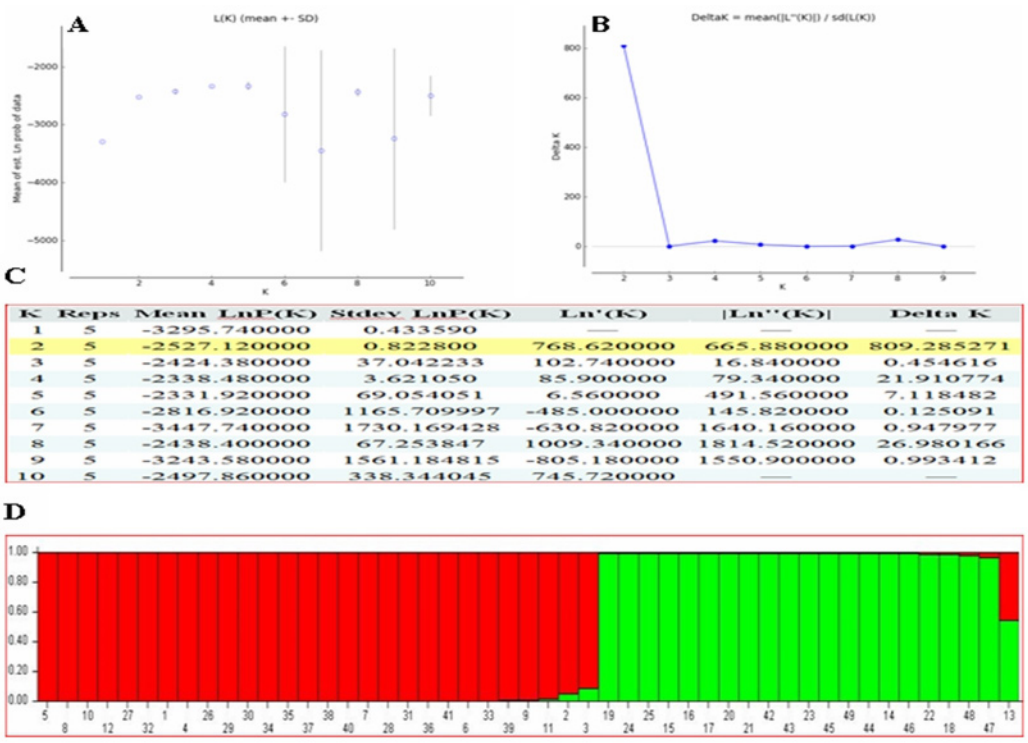


Fig. 4. Grouping of 49 bread wheat cultivars based on IRAP/REMAP data using Minimum Evolution clustering algorithm and number of difference evolutionary distance coefficient.

REMAP markers were used to construct a Minimum Evolution dendrogram which depicted two groups in studied cultivars (Fig. 4).

STRUCTURE analysis for 49 bread wheat using IRAP/REMAP data were carried out with number of clusters (*K*) ranging from one to ten and five replicate runs for all *K* values. The highest likelihood was obtained when *K* was set to four (Fig. 5A), but there was no clear pattern for forth groups (Fig. 5A). However, using the method of Evanno et al. (2005), maximal ΔK occurred at $K = 2$ (Fig. 5B), and this was considered as number of population for 49 bread wheat cultivars. The ΔK value decreased with increased *K,* and no peak of ΔK was observed at $K>2$ (Fig. 5B). The estimated ΔK value was 809.28 for 49 bread wheat cultivars, which represent two subgroups (Fig 5C). Fig. 5D shows bar plots for $K = 2$. At $K = 2$, bread wheat cultivars were divided into winter *vs.* spring cultivars. Group A (red color group) was the largest group and was comprised of 28 cultivars, whilst group B (green color group) was comprised of 21 cultivars. Clustering of bread wheat cultivars in this group included a mixture of winter and facultative types expect for Bam and Mahdavi cultivars which are in group A. These divisions were entirely reliable with those of the Minimum Evolution clustering and PCoA. Expected heterozygosity among individuals of the cluster II (=0.30) slowly was higher than the cluster I (=0.26) (data not shown).

Principal coordinate analysis (PCoA) was carried out in order to visualize the pattern of variations among the cultivars with regards to their positions on two coordinate axes. In PCoA all cultivars were labeled with different colors based on their different growing season. The first three coordinates explained 49.73, 15.56, and 11.29% of the total molecular variation. IRAP/REMAP marker systems revealed that the winter cultivars were clustered into a single groups and spring cultivars clustered in a separate groups and facultative cultivars get intermixed in these two groups (Fig. 6). The grouping pattern obtained by PCoA was to some extent similar to that of cluster analysis.



K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	5	-3295.740000	0.433590	—	—	—
2	5	-2527.120000	0.822800	768.620000	665.880000	809.285271
3	5	-2424.380000	37.042233	102.740000	16.840000	0.454616
4	5	-2338.480000	3.621050	85.900000	79.340000	21.910774
5	5	-2331.920000	69.054051	6.560000	491.560000	7.118482
6	5	-2816.920000	1165.709997	-485.000000	145.820000	0.125091
7	5	-3447.740000	1730.169428	-630.820000	1640.160000	0.947977
8	5	-2438.400000	67.253847	1009.340000	1814.520000	26.980166
9	5	-3243.580000	1561.184815	-805.180000	1550.900000	0.993412
10	5	-2497.860000	338.344045	745.720000	—	—

Fig. 5. The pattern of population structure of the 49 bread wheat (*Triticum aestivum* L.) cultivars inferred using IRAP/REMAP data. (A) Estimated LnP(D) of possible clusters *(K) from 1 to 10, (B) ΔK* based on the rate of change of LnP(D) between successive *K*, Evanno table output (C) and (D) Population structure based on *K* = 2.

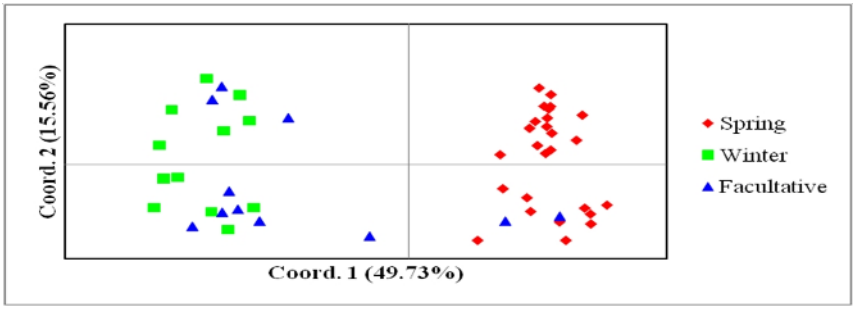


Fig. 6. Principal coordinate analysis (PCoA) of 49 bread wheat cultivars based IRAP/REMAP markers. Each cultivars is represented by one dot, with its symbol color corresponding to the assigned subgroup classification.

Discussion

RTN activity and polymorphism in bread wheat

Retrotransposon-based molecular markers including IRAP and REMAP, shed light on the genetic differentiation in bread wheat (*Triticum aestivum* L.). Retrotransposon families span boundaries between genera, meaning that retrotransposon sequences between LTR-retrotransposon plant families can be readily used across species lines, among closely related genera, and even sometimes between plant families (Kalendar et al. 2011; Lou and Chen 2007), so primers for retrotransposons originally isolated from barley, e.g. Nikita, work well as retrotransposon markers in wheat (Bento et al. 2008; Carvalho et al. 2010; Vuorinen et al. 2018). In this regard, to the best of our knowledge, only Nasri et al. (2013) used IRAP and REMAP marker systems to characterization Iranian bread wheat cultivars.

In this study, to assess insertional polymorphism in bread wheat genome, six primers designed based on the LTR sequences of the barley retrotransposon Nikita, Sukkula, and BARE-1 were tested in single and pairwise with IRAP and in combination with anchored microsatellite primers with REMAP. In IRAP analysis, four single primers namely Nikita, Sukkula, LTR6150, LTR6149, and five combinations of Nikita, Sukkula, BARE-1 primers (Nikita/LTR6150, Nikita/LTR6149, Sukkula/Nikita, Sukkula/LTR6150, Sukkula/LTR6149) produced clear and polymorphic IRAP banding patterns, indicating a more frequent activation and insertion of these retroelements in the bread wheat genome. The multiplicity

of the bands of IRAP primer Nikita generated per bread wheat cultivars supports the idea that the LTR families, tend to form local clusters in the genome of bread wheat (Nasri et al. 2013; Vicient et al. 1999). In fact, research works of Bento et al. (2008) demonstrated that LTR primer Nikita generated a high level of insertional polymorphism in bread wheat and have been active during *Triticum* species evolution. The applicability of the barley RTNs for genome analysis in the genera *Aegilops*, *Hordeum*, *Argania* and *Triticum* has been previously demonstrated (Kalendar et al. 1999; Nasri et al. 2013; Queen et al. 2004; Vuorinen et al. 2018). Vicient et al. (1999) indicated that grasses share transcriptionally, translationally, and insertionally active RTN families. When we compared the informativeness and discriminative power among the retrotransposon families, our study showed that Nikita was conserved and had relatives in the bread wheat genome and are transpositionally active, as evidenced earlier (Carvalho et al. 2010; Saeidi et al. 2008). Primer 5′LTR1 did not produce bands as a single primer in IRAP reactions, but this primer amplified bands in REMAP reactions probably proposing their presence in the bread wheat genome as solo LTRs and the preferential integration of this RTN family near SSR motifs in the wheat genome. Most of the RTNs used (expected 5′LTR2) here generated bands in REMAP reactions, showing their insertion near or in SSR motifs. In our study, the high level of polymorphism detected by both IRAP (81.78%) and REMAP (86.40%) markers may be related to the different pedigrees of the genotypes (Supplementary Table 1) and transpositionally active of the retrotransposon families used in bread wheat genome. Several studies have demonstrated the highly dynamic changes in the retrotransposon content under various biotic and abiotic stresses, thereby increasing genome size (Abdollahi Mandoulakani et al. 2014; Galindo-Gonzalez et al. 2017; Grandbastien et al. 2005; Jaaskelainen et al. 2013; Vuorinen et al. 2018). Tabrizivand Taheri et al. (2018) used the same primers for assessment of genetic diversity and relationships among *Triticum urartu* and *Triticum boeoticum* populations collected from west and northwest Iran, stated that Sukkula and Nikita retoelements showed the more prominent role in describing the genetic diversity of these species. In the all three markers system, the values level of I, He, Ne, and PPL calculated for facultative cultivars slowly were more than winter and spring cultivars in the present research. Facultative cultivars are diverse and cultivated in the most regions where growth takes place primarily in the cool, wet winters and not in the dry summers, therefore these cultivars have adapted to different climatic conditions. Since facultative cultivars commonly experience stress throughout their life cycle, activated retroelementes in response to stress induces mutations to reflect the epigenetic mechanisms. Therefore, epigenetic changes produced by the stress elicitors can help the plants adapt to new environmental conditions (Nozawa et al. 2017). Kalendar et al. (2000) stated that in Mediterranean climates retrotransposon integrational activity (specially *BARE*-1), by increasing genome size, may be adaptive.

Genetic relationships and population structure of the bread wheat cultivars

Correlations between the three cophenetic matrices generated from the IRAP, REMAP, and IRAP/REMAP dendrogram showed a relatively high and significant congruence of IRAP and REMAP with IRAP/REMAP. However, the matrices estimated by the techniques individually depicted a low and non-significant correlation (r=0.073), as it did for barley (Kalendar et al. 1999), wheat (Nasri et al. 2013), and flax (Abbasi Holasou et al. 2016) with similar results. Since REMAP primers amplified DNA regions that could not be covered by IRAP. Therefore, IRAP/REMAP data were used to reveal the cultivars between studied cultivars. Using 216 amplified IRAP/REMAP loci and cluster analysis based on ME algorithm, two groups were identified among 49 cultivars (Fig. 4). The highest cophenetic correlation coefficient (r=0.73) support that this dendrogram is a good representation of our IRAP/REMAP data. The population structure and relationship among cultivars were analyzed using Bayesian (Fig. 5). The results showed the highest peak at k=2 (Fig. 5B), suggesting that the analyzed bread wheat cultivars can be divided into two genetically distinct groups and confirmed the results obtained with ME clustering method and also it was supported by the PCoA. In the three clusters, cultivars clustered based on their growing season. Although facultative cultivars clustered in both cluster, for example Zarin (is a facultative cultivars) clustered with winter cultivars. Mohammadi et al. (2009) investigated genetic diversity of 70 bread wheat lines and cultivars from Iranian wheat breeding program using 70 SSR markers. As a result, studied genotypes were clustered in three groups that this grouping was in agreement with the pedigree information. Nasri et al. (2013) used IRAP and REMAP techniques to assess genetic diversity across 101 Iranian bread wheat, whose reported that cultivars were distributed in two separate groups, and breading lines were located in four another groups. Abdollahi Mandoulakani et al. (2017) in analysis of genetic diversity in 48 Iranian bread wheat and 49 breeding lines, stated that both Bayesian-based clustering method and neighbor-joining analysis placed 97 wheat accessions in four distinct groups. They also reported that ISSR and IRAP/REMAP markers provide powerful tools to investigate genetic relationships among wheat cultivars and lines. In PCoA of IRAP/REMAP data, winter and facultative cultivars were clustered together and spring cultivars with two facultative cultivars were mostly divided according to their growth habits. Grouping of many cultivars based on growth habits reflect the higher resolution power of IRAP and REMAP markers in grouping the local cultivars.

Low level of genetic variation (I=0.42, He=0.29, Ne=1.52) can indicate sizable effects of the annual herb, self-pollinating nature of bread wheat and creation of bottleneck effect during bread wheat selection and evaluation. With the help of IRAP and REMAP markers, Nasri et al. (2013) have also reported low level of diversity indices (I=0.5, He=0.34, and Dice similarity coefficient=0.8) in 101 Iranian bread wheat cultivars and breeding lines. Whereas Tabrizivand Taheri et

al. (2018) reported high level of mean of polymorphism information content (0.38 and 0.40) for IRAP and REMAP markers, respectively. Also, Mohammadi et al. (2009) using 70 SSR markers in 70 elite Iranian bread wheat reported high values of gene diversity and polymorphic information content (PIC) of "0.54 and 0.49", "0.70 and 0.66" and "0.70 and 0.66", for CIMMYT genotypes, exotic genotypes, and commercial cultivars, respectively. The differences between those results and our study may be due to differences in the numbers of genotypes studied with different sources and the marker systems used.

In conclusion, the above-mentioned results suggest that IRAP and REMAP markers have demonstrated great advantages, with feasible operation, and high reliability for the study genetic relationships among bread wheat cultivars. Furthermore, besides their effective employment, both of these DNA markers may furnish comparable results in assays of genetic differentiation and population structure of bread wheat cultivars. Our results showed that the genetic diversity of bread wheat is low and it is necessary to extend the genetic base of bread wheat germplasm in Iran. Besides, in the all three marker systems, gene diversity for facultative cultivars slowly were more than winter and spring cultivars, indicate that these cultivars have adapted to different climatic conditions. The use of the strong and complementary statistical methods such as ME cluster analysis and Bayesian methods proved to be useful for the determination of genetic relationships among bread wheat cultivars and for the definition of the genetic structure of this collection. These data might be very useful in the future for planning wheat breeding programs and defining strategies for germplasm conservation. Knowledge of the population structure has great importance for studies focusing on association mapping as well, which can detect correlations between phenotypes and linked markers on the basis of linkage disequilibrium (Gupta et al. 2005).

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Supplementary Table 1. Bread wheat (*Triticum aestivum* L.) cultivars used for analyses.

Cultivars	Pedigree	Biotic and abiotic stress tolerance or sensitive	Habitat[a]	Year of release	Source[b]
Arg	Inia/22-66-1	Tolerant to salt stress	S	2009	I
Zagros	Tan" S "/Vee"//Opata	Tolerant to drought stress	S	2007	-
Mahdavi	Ti/Pch/5/Mt48/3/Wt*//Nar59/Tota63/4/Mus	Tolerant to salt and cold stress	F	1995	I
Bam	Vee"s"/Nac//1-66-22	Tolerant to salt and drought stress	F	2006	I
Arta	HD2206/Hork//Bul/6/CMH80A.253/2/M2A/CML//Ald/3/Ald*4/5/BH1146/H56.71//BH1146/3/CMH78.390/4/Seri/7//Hel/3*Cno79//2*Seri 82	Sensitive to salt and drought stress	S	2011	C
Bolani	Local variety	-	S		I
Mehrgan	SISAO/ ROTSAP*2/3/NCB*4//ZUAKS	Tolerant to salt and heat	S	2014	C
Sirvan	PRL/2* PASTOR	Tolerant to drought	S	2012	C
Roshan	Local variety	Tolerant to salt and drought stress	S	1958	I
Baharan	WS-89-7	Tolerant to drought	S	2014	C
Moghan3	Luan/3/V763.23/V879.C8//Pvn/4/Picus/5/opata	Sensitive to yellow rust and tolerant to brown rust	S	2006	I
Narin	85sal/nadlA//dnavlA/3/22-66-1	Tolerant to salt stress	S	2014	I
Alvand	1-27-6275/CF1770	Tolerant to salt, drought and cold stress	F	1995	I
Azar2	Kvz/ym71//3/Maya"S"//Bb/Inia/4/Sefid	Tolerant to drought and cold stress	W	2009	I
MV17	Hungary	Tolerant to yellow rust and cold stress	W	1993	E
Gaspard	Franc	Tolerant to cold	W	1994	E
Urom	Her/Alvand//NS732	Tolerant to cold	F	2010	I
Karaj2	(Fa*Th-Mr)Omid	Tolerant to cold	F	1973	I
Soisson	-	Tolerant to cold	W	1994	E
Navid	(Kirkpinar 79) 63-112/66-2*7C	Tolerant to cold and sensitive to drought and salt	F	1990	I
Zarrin	PK15841	Tolerant to cold and sensitive to drought and salt	F	1995	E
Pishgam	Bkt/90-Zhong	Tolerant to drought	F	2008	I
Shahryar	Kvz/Ti71/3/Maya"s"//Bb/Inia/4/Karaj2/5/Anza/3/Pi/Nar//Hys	Tolerant to cold and sensitive to yellow and brown rust	W	2002	I
Zare	11,1L130//70,35F/Lira/3/Ymh/Tob//Mcd/4/Mo73	Tolerant to drought	W	2010	C
Mihan	Bkt/90-Zhong87	Tolerant to drought	W	2010	I
Arvand	Rsh(Mt-Ky*My48)	-	S	1973	I
Tajan	Bow''s''/Nkt''s'' CM67428-GM-LR-5M-3R-1LB-0Y	Sensitive to salt, yellow and brown rust	S	1995	I
Shoosh	STORK/3-*CBRD*X DICOCCOIDES	Tolerant to heat	S	2014	C
Chamran2	Attila Bacanora/ Attila//50y	Tolerant to heat	S	2013	I
Gonbad	ATRAK/WANG-SHUI-BAI	Tolerant to fungal infections	S	2013	I
Ofog	Attila/GF-gy54	Tolerant to salt	S	2012	I
Aflak	HD160/5/Cno/Tab/23854/3/Nai60//Son64/Tit/4/Son64/LR	Tolerant to heat	S	2010	C
Sivand	Kauz"S"/Azd	Tolerant to yellow and brown rust	S	2009	I
Parsi	Dove"s"/Buc"s"//2*Darab	Tolerant to yellow and brown rust	S	2009	I
Sistan	"Bank"s"/Vee"s	Tolerant to salt	S	2006	C
Hirmand	Byt/4/Jar//Cfn/Sr70/3/Jup''s''	Tolerant to salt and drought	S	1991	I
Dez	Kauz*2/Kauz//Opata	Sensitive to yellow rust and tolerant to heat	S	2002	C
Pishtaz	Alvand//Aldan/las58	Tolerant to drought and yellow and brown rust	S	2002	I
Falat	Seri82 = Kvz/Buho''s''//Kal/Bb	Tolerant to lodging and sensitive to yellow rust	S	1990	I
Niknajad	F13471/Crow''s''	Tolerant to drought sensitive to yellow rust	S	1995	I

Supplementary Table 1. Bread wheat (*Triticum aestivum* L.) cultivars used for analyses. (Continued)

Cultivars	Pedigree	Biotic and abiotic stress tolerance or sensitive	Habitat[a]	Year of release	Source[b]
Kavir	Stm/3/Kal//V534/Jit716	Tolerant to salt and drought	S	1997	I
Alamoot	KVZ/Ti71/3/Maya''s''//Bb/Inia/4/Kj2/5/Anza/3/Pi/Ndr//Hys	sensitive to salt and drought	W	1995	I
Noorestar	-	Tolerant to cold	W	1997	E
Gascojen	Gascojen	Tolerant to cold	W	1994	E
Sabalan	(21AnF*809)*1-23-2824	Tolerant to drought and cold and sensitive to salt	W	1981	I
Bisetoon	Piave*592-36-9	Tolerant to cold	W	1981	I
Kaveh	Fta-P1	Sensitive to brown, yellow and black rust	F	1980	C
Toos	*"Nzr/3/Spn/Mcd//Cama"*	Tolerant to drought and cold	F	2002	E
Heidari	Ghk"s"/Bow"s"//90Zhong87/3/Shiroodi	Tolerant to yellow rust and sensitive to brown rust	F	2006	I

[a]S, Spring; W, Winter; F, Facultative
[b]C, CIMMYT materials; E, exotic materials; I, Iranian local commercial and adapted materials.