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# Internal Transcribed Spacer (ITS) regions: A powerful tool for analysis of the diversity of wheat genotypes

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Wheat is a widely cultivated crop and it is one of the major food sources worldwide. Among the various tools used to study diversity of wheat species, the internal transcribed spacer (ITS) assessment emerges to be the more appropriate approach. In the present study, we evaluated 15 genotypes of Iranian wheat cultivars (wild, native, and breed) using ITS gene sequences. Similarity matrices and dendrogram of phylogenic relationship were constructed using Mega ver6 software. We report the major nucleotide changes in the same position between diploid and hexaploid species. dN/dS ratio for diploid, tetraploid, and hexaploid species indicated a pure selection in the examined gene, with no key changes in the genes, and 91% ITS diversity within individual wheat was evident. The results suggest that as evolution moves forward, nucleotide changes are reduced so that only a few changes in nucleotides occur. ITS marker can distinguish different wheat genotypes at the genomic level and thus prove to be the most appropriate assessment tool for analyzing inter and intra-species relationships.

**Keywords:** dN/dS ratio, Haplotype, Nucleotide Diversity, Singleton, *Triticum aestivum*

Wheat (*Triticum aestivum* L., 2*n*=42, AABBDD) is one of the major food sources worldwide. It is one of the widely cultivated crops in different parts of the world[1]. Agriculture development has spread domesticated wheat varieties across Asian, European, and African continents[2]. The North East expansion of domesticated wheat cultivation has produced sympatry with *Aegilops tauschii* with the emergence of hexaploid common wheat (*T. aestivum*)[3]. The wheat genome is allohexaploid carrying three sub-genomes, namely A, B, and D. The large genome size (16000 Mbp), the ploidy level, and the high repetitive DNA content (80%) has made positional cloning of wheat genes very complex[4].

To achieve desired genetic modification in organisms, understanding and evaluating genetic diversity is quite important[5,6]. Genetic modifications of crop plants are carried out to improve the quality of the plant with respect to its growth, nutritional value, resistance, and yield[7]. Realizing the level of genetic diversity and the relationships between genotypes is necessary to select the parents for effective crosses and adopt a suitable corrective method[8]. Many approaches have been suggested to detect diversity among wheat genomes; however, the ITS approach seems to be more appropriate as molecular markers are powerful tools for identifying cultivars, examining the evolution of species, and investigating the diversity both within and between populations[9].

Molecular markers play an essential role in all aspects of plant breeding[10]. They are neutral in terms of phenotypes and are not affected by environmental conditions, unlike morphological and isosyemic markers. Furthermore, they show some polymorphism, despite the high degree of heritability[11]. Many molecular markers such as SSRs (Simple Sequence Repeat)[12], RAPD (Random Amplification of Polymorphic DNA)[13], etc., have been used to evaluate the wheat genome. However, the information on the use of Internal Transcribed Spacer (ITS) nucleotide sequences to recognize genomic differentiation in the wheat genome offers distinct advantages over other molecular[14]. The variations in the sequences of ITS regions have been the basis of using the ITS region in phylogenetic analysis of a wide variety of organisms[15]. ITS markers

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have been used to elucidate phylogenetic relationships of monogenic wheat species and to figure out the origin and evolution of tetraploid wheat[16]. The ITS region analysis helps identify a wide variety of wheat and gives a clear barcode gap between inter-and intra-specific variation[17]. Hence, in the present study, we compared the ITS 1 and 4 nucleotide sequences of the wheat genome at three genomic levels [wild (AA), native (AABB) and breed wheat (AABBDD)] and used ITS sequences to find genetic diversity and distinguish subspecies from each other, in case sufficient variability existed for identification at the subspecies level.

## Materials and Methods

### Plant materials

Fifteen genotypes of Iranian wheat cultivars (wild, native, and breed) (Table 1)[18] were obtained from Gene bank of the university of Ilam (33°38′15″N 46°25′22″E), Iran and Zabol Agricultural Research Center (31°01′43″N 61°30′04″E), Zabol, Iran. The cultivars were evaluated for their genetic diversity using ITS gene sequences.

### DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted according to the SDS method[19], and amplification of the ITS region was carried out using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')[20] and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')[21] primers. The PCR mixture contained 20 ng DNA, 5 µL master mix (Amplicon, Denmark), 0.7 µL of each primer (10 pmol), and water to obtain a final volume of 15 µL[22]. PCR amplification was carried out on Thermal Cycler (22331 Humburg, Germany), with the thermal cycling profile of initial denaturation at 95°C for 5 min, followed by 35 cycles of 55 s at 94°C, 50 s at 56.5°C, 55 s at 72°C, and final extension at 72°C for 10 min. To ensure successful amplification, PCR products were loaded on 2% agarose gel, stained with Gel Red.

### Sequencing and analysis of ITS1-ITS4 region

Assessment of the PCR products was carried out by Chromas 2.3 software. Each of the 22 sequences was analyzed against the sequences available in the NCBI database phylogenetic evolutionary analysis was performed by using MEGA ver6 software[23]. DnaSP5 software was used to perform additional analyses and calculate dN and dS. Nucleotide substitution was also calculated based on the Tamura-Nei transitional and transversional model[24].

### Nucleotide changes in the sequence of its region at genomic levels

The nucleotide diversity of all sequences was analyzed calculating the number of polymorphic sites and Tajima's and Watterson's estimators of Theta. Tajima's *D* was also used to decipher the neural evolution of the wheat ITS gene family in the taxa. For this, Tajima's Neutrality Test[25] and the phylogenetic analysis were performed[24].

Tajima's *D*, and Fu and Li's *D**, *F**, *D* (named $D^F$) and *F* test statistics were conducted[26,27] to decipher the information only from intra-specific data, while Fu and Li's $D^F$ and *F* statistics use information from the recently evolved member and, therefore, requires the presence of an out-group to be computed. The codon usage was calculated from GenScript rare codon analysis report. Nucleotide polymorphism was measured by Theta (Θ), the number of segregating sites[28], and its standard deviation (SΘ) were estimated by Dnasp software[29].

Table 1 — Name and characteristics of wheat cultivars used in this study[18]

| Latin name | Persian Name/Origin | Accession no. | Pedigree |
|---|---|---|---|
| *Triticum aestivum* | Aflak | MF480396 | HD5/160/Tob/Cno3/23854//Nai60//Tit/Son4/64/LR/Son64 |
| *Aegilops tauschii* | Rasht/Gilan | MF480398 | Wild |
| *Triticum aestivum* | Sistan | MF480399 | Bank"s"/Vee"s" |
| *Triticum aestivum* | Kalak_Afghani/ Sistan and Baluchistan | MF480400 | Native |
| *Triticum aestivum* | Hamoon | MF480401 | Falat/Roshan |
| *Triticum aestivum* | Kavir | MF480401 | Stm/3/Kal//V534/Jit716 |
| *Triticum aestivum* | Ofogh | MF480403 | *Attila/GF-gy54* |
| *Triticum aestivum* | Bolani/ Sistan and Baluchistan | MF480404 | Native |
| *Triticum aestivum* | Arg | MG763236 | Inia/1-66-22 |
| *Triticum aestivum* | Hirmand | MF480405 | Byt/4/Jar//Cfn//Sr70/3/Jup"s |
| *Aegilops speltoides* | Galehdar/Ilam | MF480406 | Wild |
| Durum wheat | Behrang/Sistan and Baluchistan | MG757736 | Native |
| Durum wheat | Shabrang/Sistan and Baluchistan | MG757735 | Native |
| Durum wheat | Dandan_Shotori/ Sistan and Baluchistan | MG763925 | Native |
| *Triticum_urartu* | Galehdar/Ilam | MH188316 | Wild |

## Results

### Sequencing and analysis of ITS1-ITS4 region

Phylogenetic analysis of the examined ITS sequences and its comparison with the reference sequences in the NCBI revealed 99% similarity. The fragment size of the sequences was approximately 900 bp. All the sequences were deposited to NCBI GenBank under particular accession numbers (Table 1). The number of positions with and without insertion and deletion, SNP, and haplotype based on landraces (diploid), durum (tetraploid) and breed wheat (hexaploid) genome are shown in Table 2.

The total number of mutations decreased from the diploid to the hexaploid genome. Though diploid wheat species showed several sections of conserved sequences, tetraploid and hexaploid wheat species showed less number of the conserved sequences. While the total number of mutations was more at the diploid genome level than other genome levels, the frequency of loci without removal and addition were less in hexaploid genomes than other ones. The haplotype number was based on landraces (diploid) [2], durum (tetraploid) [4], and breed wheat (hexaploid) [6] genomes.

### Nucleotide changes in the sequence of its region among genome levels

Comparison of nucleotide changes among different genome levels revealed that the most nucleotide changes have occurred at the same position, with the differences between diploid and hexaploid genomes (Fig. 1A) and between tetraploid and hexaploid genomes (Fig. 1B). However, the level of nucleotide changes between diploid and hexaploid genomes was more than that of tetraploid and hexaploid genomes.

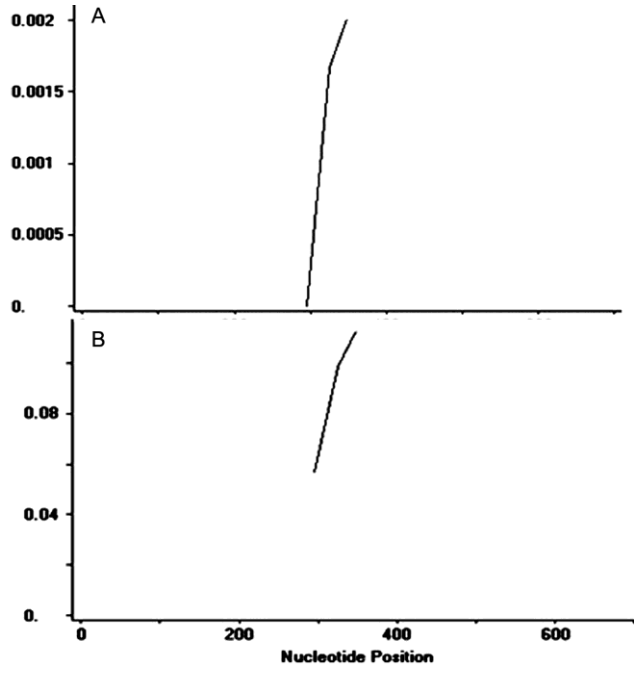


Fig. 1 — Nucleotide changes in the position of ITS region among sequences of hexaploid and (A) diploid; and (B) tetraploid

Assessment of transitional and transversionsal nucleotide substitutions, the highest and lowest minimum, transitional rates belonged to purine (18.33%) and pyrimidine (6.86%) bases, respectively, and the maximum (8.16%) and while higher (5.43%) transversionsal rates belonged to the substitution of cytosine with adenine, cytosine with guanine, thymine

Table 2 — Calculations of genetic indices based on nucleotide positions in the ITS sequence

| Genome levels | T | Eta | S | M | R | SNP | H | Hd |
|---|---|---|---|---|---|---|---|---|
| Diploid | 800 | 725 | 65 | 660 | 75 | 10 | 2 | 0.25 |
| Tetraploid | 666 | 574 | 342 | 232 | 92 | 15 | 3 | 1 |
| Hexaploid | 620 | 130 | 93 | 37 | 490 | 19 | 7 | 0.91 |
| All genome levels | 387 | 133 | 112 | 21 | 254 | 30 | 10 | 0.949 |

[T, total number of positions; Eta, total number of mutations; S, number of polymorphic sites; M, number of monomorphic site; R, loci without removal and addition; SNP, single nucleotide polymorphism; H, number of haplotypes; Hd; Haplotype diversity. T= ((Eta(S+M) +R)]

Table 3 — Maximum likelihood estimate of substitution pattern and rates matrix of ribosomal genes in wheat

| | A | T/U | C | G |
|---|---|---|---|---|
| A | - | 5.43 | 8.16 | 7.25 |
| T/U | 6.84 | - | 18.33 | 7.25 |
| C | 6.84 | 12.18 | - | 7.25 |
| G | 6.86 | 5.43 | 8.16 | - |

[Each entry is the probability of substitution (*r*) from one base (row) to another base (column). Substitution patterns and rates were estimated under the Tamura-Nei (1993) model[44]. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in *italics*]

Table 4 — The base pair ratio of ITS sequences in all landrace and cultivars of wheat

| Genotypes | T(U) | C | A | G |
|---|---|---|---|---|
| *Triticum aestivum-Aflak* | 15.6 | 29.5 | 26.1 | 28.9 |
| *Aegilops tauschii-Rasht* | 17.5 | 31.6 | 22.4 | 28.4 |
| *Triticum aestivum-Sistan* | 20.5 | 29.8 | 20.3 | 29.5 |
| *Triticum aestivum-Kalak Afghani* | 21.4 | 29.0 | 21.8 | 27.8 |
| *Triticum aestivum-Hamoon* | 23.8 | 23.8 | 29.2 | 23.1 |
| *Triticum aestivum-Kavir* | 19.5 | 22.9 | 24.3 | 33.3 |
| *Triticum aestivum-ofogh* | 23.9 | 30.1 | 16.5 | 29.4 |
| *Triticum aestivum-Bolani* | 17.6 | 31.7 | 22.3 | 28.4 |
| *Triticum aestivum-Arg* | 18.1 | 32.6 | 21.0 | 28.2 |
| *Triticum aestivum-Hirmand* | 19.1 | 29.9 | 22.1 | 28.9 |
| *Aegilops speltoides-Galehdar* | 17.6 | 32.0 | 22.2 | 28.2 |
| *durum wheat-Behrang* | 18.9 | 31.3 | 21.5 | 28.3 |
| *durum wheat-Shabrang* | 25.5 | 24.2 | 23.1 | 27.2 |
| *durum wheat-Dandan Shotori* | 25.5 | 26.1 | 23.3 | 25.2 |
| *Triticum urartu-Galehdar* | 14.8 | 32.4 | 21.4 | 31.4 |
| *Avg.* | 19.9 | 29.1 | 22.4 | 28.6 |

with adenine and thymine with guanine (Table 3). The average ratios of thymine, cytosine, adenine, and guanine in the population were obtained as 19.9, 29.1, 22.4, and 28.6%, respectively (Table 4).

Assessment of other traits such as Theta (per sequence) from Eta, Theta (per site) from Eta, Fu's Fs statistic, Strobeck's S statistic, Fu and Li's D* test statistic (–1.08108), Fu and Li's F* test statistic (–1.23930) and Tajima's D (–1.10160) were negative and insignificant (Table 5).

The numerical value of dN/dS ratio for diploid, tetraploid**,** and hexaploid genomes was 0.45, 0.59**,** and 0.52, respectively. dN/dS ratio for all genome levels was 0.13, i.e.,>1.0 indicates the occurrence of pure selection on the desired gene, without any major key changes. The results of Tajima's D test confirmed the results of the dN/dS ratio assessment.

**Comparison of genetic data between paired regions**

Analyzing the genetic distance matrix between the studied samples based on the maximum likelihood model indicated the most genetic distance at diploid genome level between *Triticum urartu* (Galehdar) and *Aegilops tauschi* (Rasht), at the tetraploid genome level (durum wheat) between Behrang and Dandan Shotori, and among breed wheat between Aflak and Ofogh cultivars (data is not shown). Among all genome levels, the highest diversity index (0.615) was found at the hexaploid genome level**,** and the least diversity index (0.007) was found in the diploid genome level. The diversity index in the tetraploid genome was observed as 0.524. These results indicated that the order of genetic diversity during the evolution period is diploid followed by tetraploid and hexaploid. Analysis of molecular variance between and among different genome levels of wheat landraces and cultivars indicated 91% variance within the population.

A Neighbor-joining phylogenetic tree based on the simple matching distance clustered 15 wheat genotypes of three different genome levels of diploid (Fig. 2A), tetraploid (Fig. 2B), and hexaploid (Fig. 2C) genotypes into two major and separate groups. *Aegilops* and *Triticum* were separated from each other and also being placed into two different clusters (Fig. 3).

The number of mutations at the diploid genome level was more than that of the tetraploid genome followed by the hexaploid genome, but the SNP was overhand concluding that as the hexaploid genome is the combination of all three genomes (AA, BB, and DD genome), it is expected that the occurrence of

Table 5 — Results from Tajima's neutrality test

| G | *n* | *S* | $p_s$ | $\Theta$ | $\pi$ | *D* |
|---|---|---|---|---|---|---|
| Diploid | 725 | 32 | 0.152381 | 0.101587 | 0.103175 | –0.77825 |
| Tetraploid | 574 | 342 | 0.595819 | 0.397213 | 0.433798 | –0.1.1253 |
| Hexaploid | 130 | 93 | 0.715385 | 0.263216 | 0.193162 | –1.372780 |
| All | 133 | 12 | 0.842105 | 0.264802 | 0.241758 | –0.388957 |

[*G*, genome level; *n*, total number of sites; *S*, No. of segregating sites; $p_s$, $S/n$; $\Theta$, $p_s/a_1$; $\pi$, nucleotide diversity; and *D*, the Tajima test statistic (chapter 12 in ref.[24] for details)]

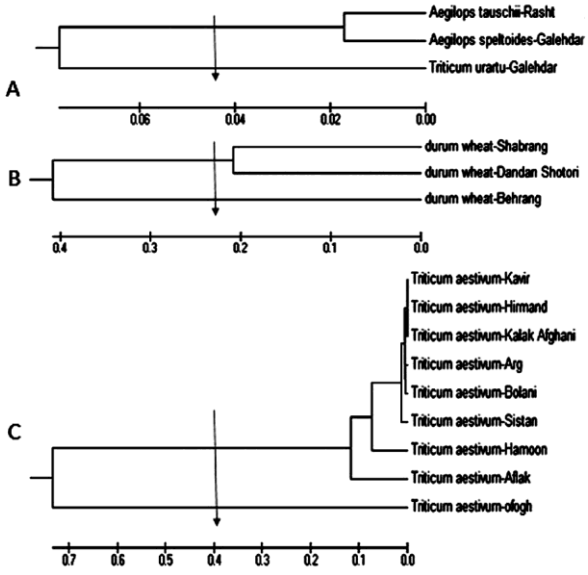


Fig. 2 — Cluster analysis of (A) diploid; (B) tetraploid; and (C) hexaploid genotypes of the wheat base on ITS sequences using Clustal W and NJ methods using MEGA6 software.

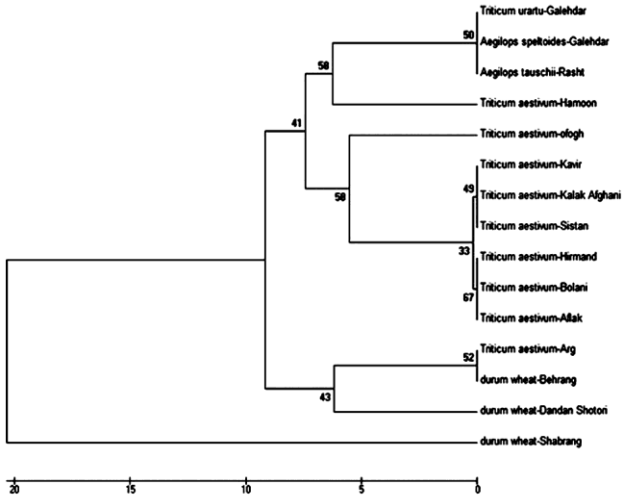


Fig. 3 — Cluster analysis of all genome levels of wheat using Muscle and UPGMA methods base on ITS sequences using Clustal W and NJ methods using MEGA6 software.

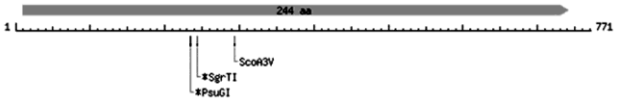


Fig. 4 — Restriction enzymes cut position on the ITS genes sequence.

several mutations in diploid and tetraploid genomes create hexaploid genome with more SNPs and new traits (bread wheat has a lot of cultivars and varieties with special SNPs and traits).

According to the results of the present study, the total number of sites per number of segregating sites was more in the hexaploid genome than in diploid and tetraploid genomes. On the other hand, the most genetic distance was observed among hexaploid genotypes as compared to diploid and tetraploid ones.

***In silico* analysis of wheat samples**

The results of the consensus sequence revealed that different human enzymes could cut the amplified area (Fig. 4), three PsuG1 restrictions enzymes with a cut site (5'….BBCGD…3' and 3'...VVGCH...5') can cut and differentiate the amplified area in different samples.

## Discussion

Since the evolution period of the hexaploid genome occurred after diploid and tetraploid genome levels, and hexaploid wheat species appeared, followed by tetraploid and diploid species, the mutation has occurred in the past, and its frequency has been decreased in the period of evolution[12]. While the total number of mutations was more at the diploid genome level than other genome levels, the frequency of loci without removal and addition were less in hexaploid genomes than other ones[31,32].

The numerical value of 1.0 for the dN/dS ratio in all cases indicated the occurrence of pure selection on the desired gene without any major changes. Tajima's D test confirmed the results of the dN/dS ratio. Our results are in line with the results of other researchers[33]. The haplotype number was based on landraces (diploid) [2], durum (tetraploid) [4]**,** and breed wheat (hexaploid) [6] genomes. Our results are in accordance with the results of previous research[15,34].

The results of nucleotide changes suggested that as evolution moves forward, nucleotide changes are reduced. Analysis of the nucleotide variations that alter the amino acids (dN) is a more useful and highly efficient method to detect the process of natural selection during genetic evolution. If the ratio is greater than one, the selection is positive; if less than one, it is a pure selection, and if equal to one, it indicates a neutral selection during the evolution of these genes[35]. The results of Tajima's D test confirmed the results of the dN/dS ratio assessment.

The GC content value for all genome levels is in accordance with the previous reports and within the ideal percentage range [30 and 70%] of GC content[33]. These results suggest that the ITS marker is an appropriate tool for analyzing inter and intra-species relationships[15,36]. The genetic diversity among inter and intra-species indicates biodiversity[15]. It can be concluded that the analysis of the ITS region is a suitable method to analyze inter and intra-species differences[15].

The number of mutations at the diploid genome level was more than that of the tetraploid genome followed by the hexaploid genome, but the SNP was overhand concluding that as the hexaploid genome is a combination of all three genomes (AA, BB, and DD genome), the occurrence of several mutations in diploid and tetraploid genomes create hexaploid genome with more SNPs and new traits (bread wheat has a lot of cultivars and varieties with special SNPs and traits)[36].

The genetic diversity may be because mutations and SNPs occurred at diploid and tetraploid genomic levels and all of them accumulated at higher genomic levels (hexaploid), and this is the reason why the higher genomic levels are more diverse[15]. Low nucleotide diversity suggests that several kinds of polymorphisms are out of conserved sequence among different wheat genome levels. Our results are similar to previous studies indicating the presence of nucleotide additively (two different nucleotides at the same locus) at several sites in the ITS region of several *Mangifera* L. species[37].

Using diploid and tetraploid genotypes as the hexaploid wheat ancestors in this research, the ITS marker can differentiate the genotypes at the genomic levels, indicating the strength of the ITS marker in the separation of species and subspecies from each other. A combination of diploid and tetraploid genotypes with the hexaploid genotype in the dendrogram can be due to various reasons[38,39] and it played a significant role in the evolution of cultivated bread wheat[39-42]. Furthermore, *Aegilops tauschii*, a wild wheat relative, is the D-genome donor of common wheat[44,45].

## Conclusion

The absence of mutation leading to the evolution of new wheat variety suggests ITS sequences as suitable

markers to estimate phylogenetic relationships within the wheat species and phylogenetically informative tools to investigate diploid, tetraploid, and hexaploid species of the wheat genome. Results on nucleotide changes in the sequence of the ITS region among different genome levels revealed the occurrence of most nucleotide changes at the same position, with the differences between diploid and hexaploid genomes and between tetraploid and hexaploid genomes. The occurrence of nucleotide changes at the same position confirms that diploid genotypes are the ancestors of tetraploid and hexaploid wheat genotypes. Assessment of genetic diversity in breeding programs plays an important role in plant improvement, i.e., development of resistance to pests, diseases, and environmental stresses.

## Conflict of Interest

Studies on human or animal subjects were not conducted.

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