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Characterization of Iranian Accessions of *Aegilpos crassa* Boiss. Using Flow Cytometry and Protein Analysis

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ABSTRACT

In this study, 120 accessions of *Aegilops crassa* collected from various geographical areas of Iran were analyzed with respect to genome size and protein markers. A flow cytometry survey of these accessions revealed that one hundred and thirteen of the accessions were tetraploid and seven were hexaploid. Moreover, these accessions revealed variations in high molecular weight glutenin subunit compositions. In most accessions, subunits showing electrophoretic mobility similar to that of Dy12 were present. Eleven allelic variants were observed in *Glu-D1* locus with the highest (30.90%) and the lowest allele (0.5%) frequencies in 3+12 and 2+10 variants, respectively. Among 17 bands selected for MALDI-TOF-TOF-MS analysis only 6 bands were identified with high probability and 11 of them had no MS/MS data. The results showed that Iranian accessions of *Ae. crassa* formed an interesting source of favorable glutenin subunits that might be very desirable in breeding programs for improving bread wheat quality.

Keywords: *Aegilops crassa*, Glutenin, Mass spectrometry, Protein, SDS-PAGE.

INTRODUCTION

The gluten proteins are the main components of wheat storage proteins, consisting of high and low molecular weight glutenin subunits and gliadins. The high molecular weight glutenin subunits (HMW-GS) account for about 25-35% of the total gluten proteins and extensively studied (Seilmeier *et al.,* 1991; Dumur *et al.,* 2009). Genetic studies revealed that the HMW-GS are encoded at several complex and highly polymorphic loci (Glu-A1, Glu-B1 and Glu-D1), located on the long arms of the homoeologous group 1 chromosomes of wheat (Payne *et al.,* 1983). Each locus consists of two tightly linked genes that encode for a higher molecular mass subunit and a lower molecular mass subunit, which are termed *x*- and *y*-type subunits, respectively. In addition, there are also allelic variations in the structures and properties of the subunits encoded by the genes. These subunits can be recognized and numbered in accordance with their electrophoretic mobility in SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). This class of gluten proteins is mainly responsible for dough strength in wheat, determining the quality characteristics of the dough and its end use products (Ram, 2003).

SDS-PAGE is routinely used in many studies for the selection of wheat cultivars associated with superior quality characteristics. This technique is fast and offers a very high resolution; however, it is only descriptive and gives no structural information on the protein

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components. These limitations can be overcome using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF-TOF-MS) in genomics and proteomics fields. Analysis by MALDI-TOF-TOF-MS delivers fast determination of protein molecular mass up to 100 kDa, applied to identify glutenin and gliadins in flour and food samples (Amiour *et al.,* 2003). For example, MALDI-TOF-TOF-MS was applied for the characterization of wheat HMW GS and gliadins, genetic variants, and to varietal analysis through measurement of accurate molecular weight.

As the superior HMW subunits identified in common wheat are very limited (Ruili *et al.,* 2001). Therefore it is necessary to find new HMW subunits from alternative genetic resources. *Ae. crassa* posses a *D* genome component as *Triticum aestivum* L. (AABBDD) and may be originally derived from a common ancestor (*Aegilops tauschii* Coss.) (Peterson *et al.,* 2006). Little is known about the composition of HMW glutenin subunits and ploidy levels of *Aegilops crassa* from Iran. Newly developed analytical tools such as flow cytometry can help to determine ploidy levels rapidly in closely related species. In the present study, SDS-PAGE coupled with flow cytometry led to the identification of HMW-GS compositions of 120 accessions of *Ae. crassa.*

MATERIALS AND METHODS

Plant Materials

A total of 120 accessions of *Ae. crassa* collected from different places of Iran and provided by the National Plant Gene Bank of Iran were used (Table 1).

Flow Cytometry Analyses

The C-value of each accession was estimated using flow cytometry. Nuclear suspensions were obtained from 0.5 cm^2 pieces of young leaves according to the protocol described by Galbraith *et al.* (1983) with some modifications. *Ae. tauschii* leaves with a *2C* value of 10.5 pg DNA (Lee *et al.,* 2004) were used as internal reference standard. The suspension of nuclei was analyzed by the CA-III flow cytometer with a 100W high-pressure mercury lamp, KG1, BG38, UG1 filters, TK420 dichroic mirror and a GG435 barrier filter. Data were analyzed on DPAC software (Partec GmbH, MuÈ nster, Germany). In each sample, fluorescence and scatter properties of 5,000–20,000 nuclei were assayed. The absolute DNA amount of a sample was calculated based on the values of the G1 peak means:

Sample 2C DNA content= [(Sample G1 peak mean)/(Standard G1 peak mean)]×Standard 2C DNA content (pg DNA).

Relative DNA content of individual plants was expressed using a DNA index (DI) calculated according to the following formula:

$$\text{DI}=\frac{\text{Mean of the relative DNA content of the G1/G2 nuclei of the sample}}{\text{Mean of the relative DNA content of the G1/G2 nuclei of standard}}$$

(Kantartzi *et al.,* 2010)

SDS-PAGE Analysis

Embryos were removed from each sample and the seeds were finely crushed. The flour was mixed in an extraction buffer of 0.125M Tris-HCl (pH= 6.8), buffer 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.03% bromophenol blue and 5% 2-mercaptoethanol. Samples were boiled for 5 minutes at 95°C and then centrifuged for 10 minutes at 10,000 rpm. Samples were fractionated by SDS-PAGE according to the method described by Payne and Lawrence (1983) using stacking and separating gels containing 4% acrylamide, 0.3% bis

Table 1. Geographical distribution of accessions studied.

Origin	Number of accessions	Origin	Number of accessions
Hamedan	8	Kermanshah	26
Ilam	9	Azarbaijan S	2
Azarbaijan G	26	Khorasan S	3
Zanjan	4	Markazi	2
Ghazvin	2	Khozestan	1
Fars	8	Alborz	1
Lorestan	3	Hormozgan	2
Kordestan	5	Chaharmohale B	3
Khorasan R	8	Unknwon	7

acrylamide, 10% SDS and 0.125M Tris-HCl (Ph= 6.8), and 14% acrylamide, 0.03% bis acrylamide, 10% SDS, and 0.125M Tris-HCl (pH= 8.8), respectively. Gels were stained overnight with 0.13% Comassie Brilliant blue (CBB. Sigma, USA) and then destained overnight in distilled water. After electrophoresis of seed storage proteins by SDS-PAGE, subunit mobility of HMW of *Ae. crassa* accessions was compared with three Australian standard cultivars named: Sunvale, Sunlin (encoding subunits 2Dx and Dy12) and Grebe (encoding subunits 5Dx and 10Dy) as controls.

Analysis of Selected Protein Bands with MALDI-TOF-TOF-MS

Seventeen protein bands showing polymorphism in samples were excised and analyzed using an Applied Biosystems 4,700 Proteomics Analyzer at the Protein and Proteomics Center of the National University of Singapore. Proteins were then analyzed using MALDI-TOF/TOF-MS (Peng *et al.,* 2009). Protein Database search was carried out using MASCOT Program at http://www.matrixscience.com).

RESULTS

Flow Cytometry Analyses

Results obtained from flow cytometry analysis indicated that 93% (113 out of 120) of the accessions were tetraploid, and only 6% were hexaploid (Figure 1). The coefficient of variation (CV) for $G_{0/1}$ peak varied between 2.5 and 3.8 throughout this study. Genome sizes of tetraploid and

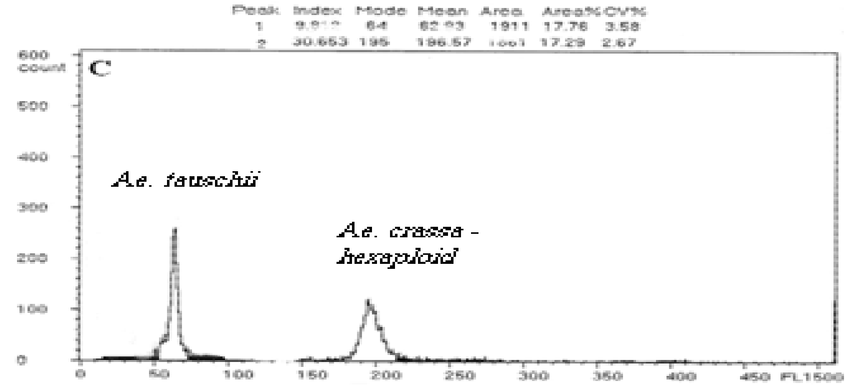

Figure 1. Histogram of relative nuclear DNA content: (A) The nuclei isolated from young leaf of *Ae. tauschii* (standard sample); (B) The nuclei isolated from young leaf of *Ae. tauschii* and *Ae. crassa* tetraploid, (C) The nuclie isolated from young leaf of *Ae. tauschii* and *Ae. crassa* hexaploid.

hexaploid accessions of *Ae. crassa* along with reference genome, *Ae. tauschii,* are listed in Table 2. Hexaploid and tetraploid accessions had mean 2c values of 31.8 and 21.7 pg, respectively. Moreover, the mean *DI* values were 3.03 and 2.07 for hexaploid and tetraploid accessions, respectively. The amount of DNA in tetraploid accessions was larger than the expected value, and the deviation was significant ($P \leq 0.0001$); while the amount of DNA in hexaploid accessions was smaller than the expected value with a significant deviation ($P \leq 0.0001$) (Table 2).

SDS-PAGE Analysis

Storage protein analysis showed a considerable genetic diversity in studied accessions. Eight allelic variants were observed at the *Glu-D1* locus with the highest (37.23%) and the lowest allele (2.1%) frequencies in 3+12 and 2+10 variants, respectively (Figure 2). The result of SDS-PAGE analysis showed that bands number 3 and 4 had similar electrophoretic mobility to subunit *Dy12* in Sunveil and Sunbre; therefore they could be a *y* type HMW glutenin subunit (Figure 3). Moreover, based on Sunveil and Sunbre band (Known as 2DX) it could be concluded that subunit number 1 could be *2DX* or a similar allele of *X*-type HMW. As the top band in Gribe is a *Dx5* subunit and therefore bands with similar electrophoretic mobility in line 2 (AC2), as indicated by number 3, could be *Dx5*. Moreover, subunit 10 in cultivar Gribe has a slightly higher molecular weight than subunit *DY12* in Sunveil and Sunbre. As indicated in this figure all bands with the same mobility such as band number 9 could be subunit *Dy10*. As it is clear in controls (Sunveil and Sunbre) the top band is subunit *1Ax*. Therefore the bands with the same mobility as this band in the other lanes could be named as *1AX*.

However, in the top of the lane AC5, AC6, AC8 and AC9 there is an unknown band with unusual HMW mobility which was located in the top compared to *1Ax*. Moreover, six new alleles likely belong to *M* genome and one new allele belongs to *D* genome were observed (Figure 3). All of these new bands along with 11 references bands were selected for analyses with MALDI-TOF-TOF-MS.

MALDI-TOF-TOF-MS Analysis

Seventeen bands were selected and digested with trypsin, and the resulting peptides were mass analyzed. These data were compared with expected values computed from sequence database entries. Among 17 bands analyzed by MALDI-TOF-TOF-MS, only 6 bands were identified with a statistically significant score. In this case, proteins hitting with a score greater than 75 are found to be significant meaning that the probability of this being a trustable hit is high. However, 11 of these bands did not show any MS/MS data (Table 3). Six identified bands were: beta-amylase similar

Table 2. Genome size, DNA Index and mean DNA content in tetraploid and hexaploid accessions of *Ae. crassa* and *Ae. tauschii* as a reference .

Species	Chromosome number (2n)	Ploidy level	Genome	*DI*	Observed mean DNA content (pg $2c^{-1}$)	Excepted mean DNA content (pg $2c^{-1}$)[b]	Genome size
Ae. Crassa	42	6	$D_1D_1MMD_2D_2$	3.03	31.8	32.06	1.5×10^4
Ae. crassa	28	4	D_1D_1MM	2.07	21.7	21.56	1.0×10^4
Ae. tauschii	14	2	*DD*	----	10.5[a]	---	4.9×10^{3a}

[a] The *2C* genome size (4.9×10^3 Mbp) and Mean DNA content (10.5 pg $2c^{-1}$) of *Ae. tauschii* (Lee *et al.,* 2004). The means were calculated from three duplicates per accession.

[b] Excepted mean DNA content (pg $2c^{-1}$) from the sum of the DNA amounts of the two parental species (see Eilam *et al.,* 2007 and Lee *et al.,* 2004 for *2C* DNA amount of and *Ae. Comosa* and *Ae. tauschii*).

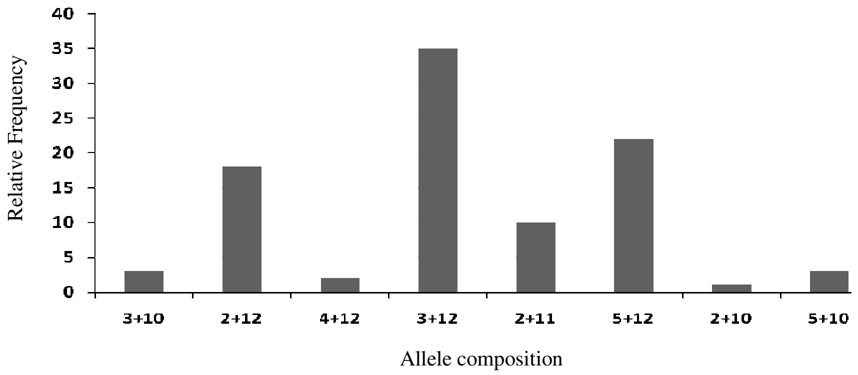

Figure 2. Composition of HMW glutenin subunits in *Aegilops crassa* accessions.

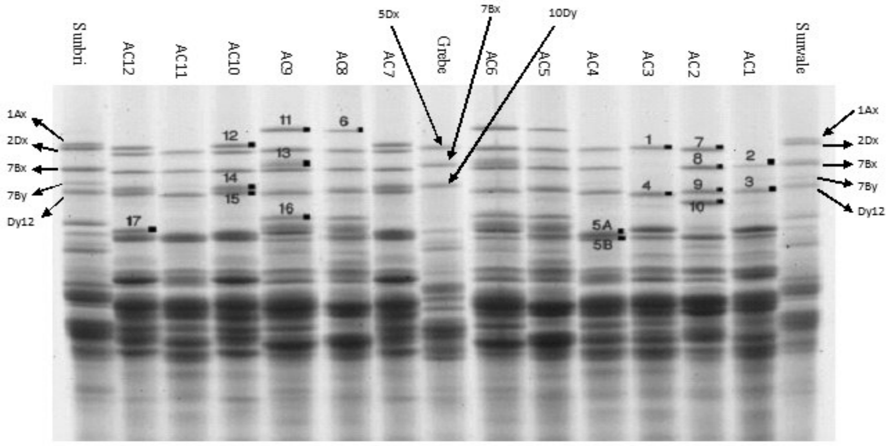

Figure 3. A sample of SDS-PAGE analysis of the composition of HMW glutenin subunits in some the *Aegilos crassa* accessions (Acc1 to ACC 12).

to *Hordeum vulgare* L. (5A and 5B), *y*-type high molecular weight glutenin subunit similar to *Aegilops ventricosa* Tausch (bands number 3 and 4) and high molecular weight glutenin subunit similar to *T. aestivum* (bands number 9 and 14) (Table 3).

DISCUSSION

Results obtained from flow cytometry analysis indicated that most of the *Ae. crassa* accessions have tetraploid genome (93%). Similar to what was obtained here,

Table 3. List of identified glutenin subunits protein including name of gene by using MALDI-TOF-MS coupled to bioinformatics.

BN[a]	Identified protein	Mr[b]	Species	Gene identifier or Swiss-Prot/NCBI accession no.	Score
18	*Beta*-amylase	56505	*Hordeum vulgare*	6729696	143
5	*Beta*-amylase	59634	*Hordeum vulgare*	10953875	117
3	HMW-glutenin subunit	19968	*Aegilops ventricosa*	7188718	75
4	HMW-glutenin subunit	19968	*Aegilops ventricosa*	7188718	86
9	HMW-glutenin subunit	20193	*Triticum aestivum*	24474926	155
14	HMW-glutenin subunit	20193	*Triticum aestivum*	24474926	147

[a] Nand Number; [b] Mass range.

previous studies (Badaeva *et al.,* 1998; Ciaffi *et al.,* 2000; Kole, 2011) also found the occurrence of hexa and tetra levels of polyploidy in *Ae. crassa*. Our results do not support the idea that all strains of *Ae. crassa* from Iran are tetraploid (Kihara *et al*., 1965).

The estimated nuclear DNA amount, 21.7 pg, for tetraploid accessions in our study seems quite close to the result of Eilam *et al.* (2008) (2C= 21.72). Our results showed that the amount of nuclear DNA is larger than the expected value, which is in agreement with previous findings that DNA content in hexaploid wheat and in most allopolyploid species of *Aegilops* and allohexaploid *Triticum* are less than expected from the amounts in the parental species (Upadhya and Swaminathan, 1963, Lee *et al.,* 2004, Eilam *et al.,* 2008). Likewise, the observed DNA amount in cytotype hexaploid is 31.8 pg, 0.81% less than the expected amount (32.06 pg). On the other hand, DNA amount estimated in hexaploid accessions of *Ae. crassa* was less than DNA content in hexaploid wheat. These interesting results might be derived from the different evolutionary process of D_1D_1, *MM,* D_1D_1MM or D_2D_2 genomes, different parental backgrounds of cytotypes *Ae. crassa* in coding and non-coding sequences or elimination of genomic or chromosome-specific sequences during the polyploidy formation.

By using conventional (SDS-PAGE) approach, coupled with MS identification of the products, we obtained complementary views of the high molecular weight glutenin subunits in *Ae. crassa.* SDS-PAGE analysis showed large variations at the *Glu-D1* locus, as was also reported using morphological (Ranjbar *et al.,* 2007) and molecular (Naghavi *et al.,* 2009) data, indicating as a suitable source of gene pool for pre-breeding and future wheat breeding programs. Since the *D* genome has greatly improved breadmaking capabilities of wheat (Dworschak *et al.,* 1992), the large variation existing in the gene pool of *Ae. crassa,* offers an attractive way to improve seed storage proteins in bread wheat.

Moreover, in common with *Ae. tauschii*, multiple subunits were expressed in the seeds of different *Aegilops* species. In most accessions subunits showing electrophoretic mobility similar to that of *Dy12* were present. These results suggest that the *D*-genome component in the multiploid *Ae. crassa* express at least one HMW glutenin subunit that is structurally related to the *1Dx* subunits of bread wheat. It is showed that allelic variation in the subunits encoded by chromosome *1D* was associated with differences in the breadmaking quality of bread wheats (Payne *et al.,* 1981).

Although, most HMW glutenin subunits have been identified and classified by SDS-PAGE based upon electrophoretic mobility, a size fractionation technique such as SDS-PAGE does not provide accurate mass values (Bunce *et al.,* 1985). Among 17 bands analyzed by MALDI-TOF-TOF-MS, only 6 bands were identified with high probability (Table 1), and 11 of them had no MS/MS data. Although they could be identified by PMF data, their identities need to be further confirmed. A top band, as indicated No. 6, in some accessions such as line AC5, AC6, AC8 and AC9 was observed. This top band is a HMW glutenin subunit with unusual HMW mobility, as expectation of HMW derived from D genome in top band in SDS-PAGE in hexaploid wheat was rare. However, Nakamura (2003) reported an unusual HMW from *D* genome estimated around 120 KD encoded from the designated locus known glu-d1f allele. This could confirm that allelic variability in *D* genome is higher than expected. Moreover, we deduced that null alleles and/or gene silencing might also affect the expression of HMW glutenin subunit genes in some accessions of *Aegilops* species. It is expected that the other bands indicated as number 10 and 16 could be derived from M genome, but mass spectrometry was not able to reveal any data for confirmation.

In view of the increasing interest in HMW glutenin subunits associated with their potential important relationship with wheat protein quality (Payne *et al.*, 1981; Ram, 2003) using MALDI may prove useful for the further characterization of individual components. Previous results indicated the feasibility of using MALDI to obtain a rapid and complete profile of HMW glutenin subunits (Dworschak *et al.,* 1998). This technique may prove particularly useful in wheat breeding programs where rapid isolation of lines containing subunits associated with superior quality is a major objective. The results obtained from this study can be useful for better management of germplasm collections.

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بررسی خصوصیات نمونه های آجیلوپس کراسا ایران با استفاده از فلوسایتومتری و آنالیز پروتئین

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چکیده

در این مطالعه، ۱۲۰ نمونه آجیلوپس کراسا جمع آوری شده از مناطق مختلف جغرافیایی ایران بر اساس اندازه ژنوم و مارکر های مولکولی مورد بررسی قرار گرفت. آنالیز فلوسایتومتری این نمونه ها نشان داد که ۱۱۳ از نمونه ها تتراپلوئید و ۷ نمونه هگزاپلوئید بوده اند. علاوه بر این، این نمونه ها تفاوت در وزن مولکولی بالا ترکیبات زیرواحد گلوتنین نشان دادند. در بیشتر نمونه ها زیر واحد های با میزان مهاجرت باندی مشابه با زیر واحد Dy12 مشاهده گردیدند.. ۱۱ الل در لوکس Glu-D1 مشاهده شد که به ترتیب بیشترین فراوانی(۳۰.۹٪) و کمترین فراوانی(۰.۵٪) مربوط به واریانت های ۱۲+۳ و ۱۰+۲ می باشد. بین ۱۷ باند انتخاب شده برای آنالیز MALDI-TOF-TOF-MS فقط ۶ باند با احتمال بالا شنتاسایی شده و اطلاعات MS/MS برای ۱۱ باند دیگر مشاهده نشد. نتایج نشان داد نمونه های آجیلوپس کراسا بومی ایران یک منبع جالبی از زیر واحد های گلوتنین مناسب را تشکل داده که ممکن است در برنامه های اصلاحی برای بهبود کیفیت گندم نان خیلی مناسب باشند.