# PLOIDY DETERMINATION OF AEGILOPS CYLINDRICA HOST ACCESSIONS OF IRAN BY USING FLOW CYTOMETRY AND CHROMOSOME COUNTING

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Chromosomes counting and flow cytometry method were used for assessing the ploidy level of various *Aegilops cylindrica* Host (2n=4x=28, CCDD) accessions of the National Plant Gene Bank of Iran (NPGBI). About 100 different accessions of *Ae. cylindrica* were estimated in flow cytometry analysis by using *Ae. tauschii* as a control sample. All the studied accessions fitted in 4*x* ploidy level. Chromosomes counts and flow cytometry data led to the same results. There was significant diversity in DNA index among *Ae. cylindrica* accessions. Cluster analysis was used for the classification of *Ae. cylindrica* based on their DNA content. Interestingly, accessions with smaller and larger DNA index had smaller and larger chromosome length, respectively. Also, the study of the relationship of DNA content and geographical distribution in Iran showed that there is diversity in DNA content of *Ae. cylindrica* for different areas of Iran.

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**تعیین سطح پلوئیدی گونه Aegilops cylindrica در ایران از طریق فلوسایتومتری و شمارش کروموزومی**

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در این تحقیق از شمارش کروموزومی و روش فلوسیتومتری برای تخمین سطح پلوئیدی نمونه های مختلف *Aegilops cylindrica* Host (2*n* = 4*x* = 28, CCDD) موجود در بانک ژن گیاهی ملی ایران استفاده شد. تعداد ۱۰۰ نمونه *Ae. cylindrica* در آنالیز فلوسیتومتری با استفاده از *Ae. tauschii* به عنوان نمونه کنترل مورد استفاده قرار گرفتند. تمام نمونه های مورد بررسی دارای سطح پلوئیدی 4x بودند. نتایج حاصل از فلوسیتومتری و شمارش کروموزومی مشابه بود. تنوع معنی داری در شاخص DNA نمونه های *Ae. cylindrica* وجود داشت. تجزیه خوشه ای برای طبقه بندی *Ae. cylindrica* بر اساس محتوای DNA آنها انجام شد. به طور جالبی، نمونه های با شاخص DNA کوچک و بزرگ به ترتیب دارای طول کروموزوم کوتاه و طویل بودند. همچنین، مطالعه رابطه بین محتوای DNA و پراکنش جغرافیایی در ایران نشان داد که برای *Ae. cylindrica* در مناطق مختلف ایران از لحاظ محتوای DNA تنوع وجود دارد.

## Introduction

*Aegilops cylindrica* Host (2*n* = 4*x* = 28, CCDD) tetraploid wild species relative with bread wheat *Triticum aestivum* L. (2*n* = 6*x* = 42, AABBDD), is native to the Mediterranean, Middle East, Asia, and was also introduced to the Great Plains and the Pacific

northwest of the United States (Kimber & Feldman 1987; van Slageren 1994). The genomic constitution of *Ae. cylindrica* was determined by the analyses of chromosome pairing (Sax & Sax 1924; Kihara 1931; Kihara & Matsumura 1941; Sears 1944; McFadden & Sears 1946), storage proteins (Johnson 1967; Masci *et al*. 1992), isozymes (Jaaska 1981; Nakai 1981), and differences in restriction length patterns of repeated nucleotide sequences (Dubcovsky & Dvorak 1994). These studies identified the diploid species *Ae. caudata* L. ($2n = 2x = 14$, CC) as the donor of the C genome and *Ae. tauschii* Coss. ($2n = 2x = 14$, DD) as the donor of the D genome of *Ae. cylindrica.* The cytoplasm of *Ae. cylindrica* was contributed by *Ae. tauschii* (Maan 1976; Tsunewaki 1989).

Microscopic chromosome counting technique in root tip cells has been conventionally conducted to determine the ploidy level, but this process is laborious and time-consuming. Recently flow cytometry (FCM) analysis has been alternatively applied for determining the ploidy level in a number of plant species. This method has the accuracy advantages, convenience, simplicity, low cost and rapidly as compared with conventional chromosome counting (Galbraith *et al*. 1983; Arumuganathan & Earle 1991; Dolezel 1997), and thus a large number of samples can be analyzed in a short period. Because of these advantages, DNA flow cytometry has been used extensively in researches to detect aneuploidy (Kawara *et al*. 1999) and monitor cell cycle kinetics and its perturbations (Rabinovitch 1994). These analyses are based on the use of DNA-specific fluorochromes and analysis of the relative fluorescence of stained nuclei (Dolezel 1991). 4, 6-diamidino-2-phenylindole (DAPI) is a fluorochrome that is widely used for DNA staining in flow cytometry of cell nuclei. Because fluorescence of the unbound stain is relatively low, background problems are less. This is important, since instruments that can use DAPI as the optimal fluorochrome are less expensive and the staining procedure is less demanding. DAPI became popular, presumably due to two important reasons: (1) DAPI is specific for double-stranded DNA and its binding to DNA is not influenced by chromatin structure, which results in low peak CVs (Cowden and Curtis, 1981); (2) many plant scientists preferred using arc-lamp-based flow cytometers, with which DAPI fluorescence was particularly easy to excite and measure. However, because of it's AT preference, estimates strongly depend on the base composition and this varies widely throughout the plant kingdom. So it can be used when comparing DNA contents of samples with the same AT/GC ratio.

In most plants, analyses of relative DNA content of nuclei isolated from young tissues, yield a histogram showing a dominant peak corresponding to nuclei at the G0/G1 phase of the cell. To estimate ploidy levels, the position of the G1 peak on a histogram of an unknown sample is compared to that of a reference plant with known ploidy (Dolezel 1997). However, the use of flow cytometry to infer ploidy level is only appropriate when comparing accessions from the same or closely related species, where large differences in chromosome size are not expected.

Compaction of DNA in polyploid nuclei can produce an underestimate of the DNA measurements, but it has also been observed in several cases where polyploids have smaller chromosomes and lower DNA content than expected (Yamaguchi & Tsunoda 1969; Martinez & Ginzo 1985; Poggio & Hunziker 1986). Vogel *et al*. (1996) used flow cytometry to determine the base DNA content of the genomes of the perennial *Triticeae* and they concluded that gain or loss of nuclear DNA content occurred during the evolution of the perennial *Triticeae* and was probably a part of speciation. Lee *et al*. (2004) used flow cytometry and chromosome imaging method for analyzing genome content and chromosomal DNA content of hexaploid wheat (AABBDD), hexaploid triticale (AABBRR), tetraploid wheat (AABB), and AA, BB, DD genome donors. The nuclear DNA content of BB genome donor was the highest value among the other genome donors, AA or DD. The genome content of tetraploid wheat, unlike hexaploid wheat or hexaploid triticale was larger than the sum of the genomes of AA and BB genome donors.

Iran is one of the diversity centers of *Ae. cylindrica* (van Slageren 1994). Therefore, it is very important to determine the ploidy levels of this species in Iran. The aim of current research is to develop an efficient system for the rapid detection of ploidy level in *Ae. cylindrica.* The accessions were characterized by DNA flow cytometry and compared its usefulness with chromosome count.

## Materials and Methods

*Plant material:* 100 accessions of *Ae. cylindrica* were used in this experiment, which were collected from National Plant Gene Bank of Iran (NPGBI). These accessions were collected from seventeen provinces of Iran (West Azerbaijan, East Azerbaijan, Ardebil, Zanjan, Qazvin, Kurdistan, Hamedan, Kermanshah, Ilam, Lorestan, Chaharmohal Bakhtiari, Mazandaran, Tehran, Esfahan, Fars, Semnan and Khorasan).

*Flow cytometric analysis:* These 100 accessions were selected from 359 accessions by cluster analysis based on morphological traits and cultured in Petri dish, so that 10 seeds were cultured per Petri dish and for evaluating of DNA content five fresh leaves were

elected. One centimeter was cut from each of selected leaf and all parts of the selected leaves were used for evaluating. According to the technique, adapted from Dolezel *et al*. (1989), leaf tissues of the analyzed samples, together with leaf tissues of standard species, were chopped by a razor blade in plastic Petri dish containing a few drops of Partec buffer, containing 4$\mu g$/ml 4, 6-diamidino-2-phenylindole (DAPI). Flow cytometric estimation of nuclear DNA content was performed with a Partec PA flow cytometer. In each sample, 70,000-100,000 nuclei were analyzed. Mode of DNA peak of G1 phase of all accessions was measured by flow cytometry with coefficients of variation (CV) usually below 5.0%. The DNA content peaks were analyzed, using the free WinMDI software (Joseph Trotter 1998).

*Control sample:* In order to eliminate differences in signal intensities due to light absorption, quenching and other variables, a piece of *Ae. tauschii* (2n=2x=14) as a control sample, with the material to be analyzed were always chopped together for 120-140 s. *Ae. tauschii* (2n=2x=14) was chosen as the control sample, because of its genetic similarity to *Ae. cylindrica*. Furthermore, there isn't significant difference in chromosome size and finally these two species share the D Genome in common. Also, Mode of DNA peak of G1 phase of *Ae. tauschii* was used to calculate the DNA index (DI) of accessions. Relative DNA content of accessions was expressed using a DNA index calculated according to the following formula:

$$DI = \frac{\text{Mode of the G1 DNA peak of } \textit{Ae. cylindrica}}{\text{Mode of the G1 DNA peak of } \textit{Ae. tauschii}}$$

*Chromosome counting:* According to the technique, adapted from CYMIIT institute (Mujeeb-Kazi 1985), root tips were collected between 09 AM to 10: 30 AM, and then placed in a Petri dish, on a filter paper moistened with α-bromonaphthalene pre-treatment solution. The samples were pre-treated about 2.5 to 3.5 hours, but generally 3 hours as pre-treatment time which was used in this study, concludes satisfying chromosome contraction and high mitotic index. After pre-treatment, the root tips were transferred to vials, containing 0.2% aceto-orcein and refrigerated (4°C), until being used. Afterwards, in order to intensify the staining for 2 days before squashing, the root tips were transferred to 2% aceto-orcein. After staining, the aceto-orcein was removed from the vial and enough 45% acetic acid was added to fill about a quarter of the vial. The vial was heated over a flame to bring the contents to a slow boiling. After boiling, the vial contents (45% acetic acid + root tip) were transferred into an evaporating dish. A root tip was taken from it and placed over on filter paper to remove extra 45% acetic acid. The 2 - 2.5 mm apical root tip was cut and placed on dry microscope slide. The root tip was squashed by an arrow-head needle, and a small drop of 45% acetic acid was quickly added to the squashed tissue. The slide was then slightly warmed and a cover glass was placed gently over on the macerated cellular area. The cover glass slides were gently dabbed with coarse filter paper, heated slightly, placed between folded filter paper on a flat surface and thumb pressure applied directly to the cover glass. After squashing, the slide was suitable for watching chromosomes in microscope.

*Statistical analysis.* In both species (*Ae. cylindrica* and *Ae. tauschii*), nuclear DNA content were analyzed using following parameters: Mean, Standard Error of Mean**,** Median**,** Mode**,** Standard Deviation, Variance**,** Skewness**,** Kurtosis, Range**,** Minimum and Maximum. Also, cluster analysis was used for the classification of DNA peak mode of *Ae. cylindrica* accessions by using Ward method. All statistical methods were performed using SPSS software (SPSS Inc., 2001)

## Results and Discussion

*DNA index, nuclear DNA content and chromosome counting.* DNA peak mode of 100 accessions of *Ae. cylindrica* were determined using flow cytometry. Significant diversity was found in DNA peak mode among *Ae. cylindrica* accessions. Also, distribution of DNA index estimating for *Ae. cylindrica* was continuous (fig.1).

Ploidy levels of *Ae. cylindrica* accessions were determined by comparing their DNA peak mode with DNA peak mode of *Ae. tauschii* (2n=2x=14 DD) as control accession for predicting ploidy level of *Ae. cylindrica*. DNA peak mode of *Ae. cylindrica* were two times bigger than *Ae. tauschii* (fig. 2).

The accessions were supposed to be Tetraploid Ploidy level. The accessions with DNA contents, which were similar in content to Tetraploid Ploidy level, predicted as potential for aneuploids, presence of B chromosomes or difference on their length of chromosomes. Mean, median and mode of DNA peak of *Ae. cylindrica* indicated that *Ae. cylindrica* is a tetraploid species but variance and rang of DNA peak of accessions showed that accessions are different with each other (table1). Also, chromosome counting showed that *Ae. cylindrica* is tetraploid ($2n = 4x = 28$) and ploidy level, measured by using flow cytometry analysis, was confirmed by chromosome counting. Furthermore, cytogenetic studies showed no aneuploids

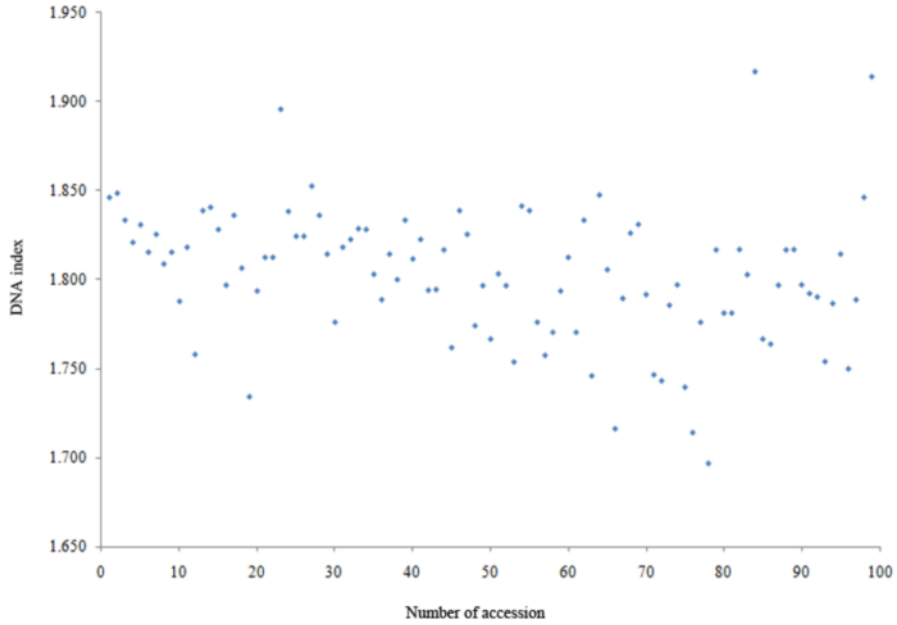


Fig. 1: Dot plot graph of DNA index content of 100 accessions of *Aegilops cylindrical* as measured by flow cytometry.

Table1. Statistical parameters for nuclear DNA content (G1 phase) of *Aegilops cylindrica*.

| Parameter | Value |
|---|---|
| Mean | 118.39 |
| Standard Error of Mean | 0.874 |
| Median | 117.00 |
| Mode | 127 |
| Standard Deviation | 8.696 |
| Variance | 75.629 |
| Skewness | 0.268 |
| Kurtosis | -0.543 |
| Range | 42 |
| Minimum | 96 |
| Maximum | 138 |

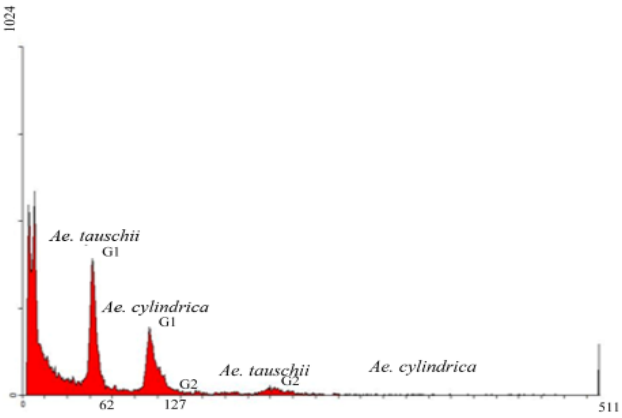


Fig. 2. Histogram of DNA content obtained from flow cytometric analysis of *Aegilops tauschii* and *Ae. cylindrica*.

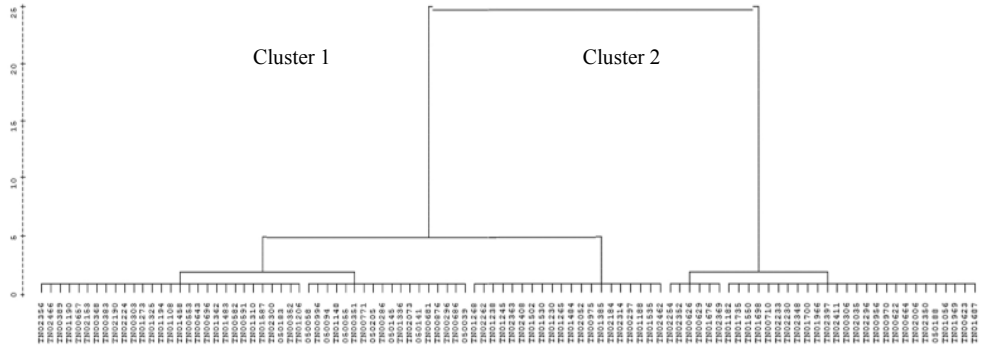


Fig. 3. Cluster analysis of all accessions of *Aegilops cylindrica* for their DNA peak mode of G1 phase.

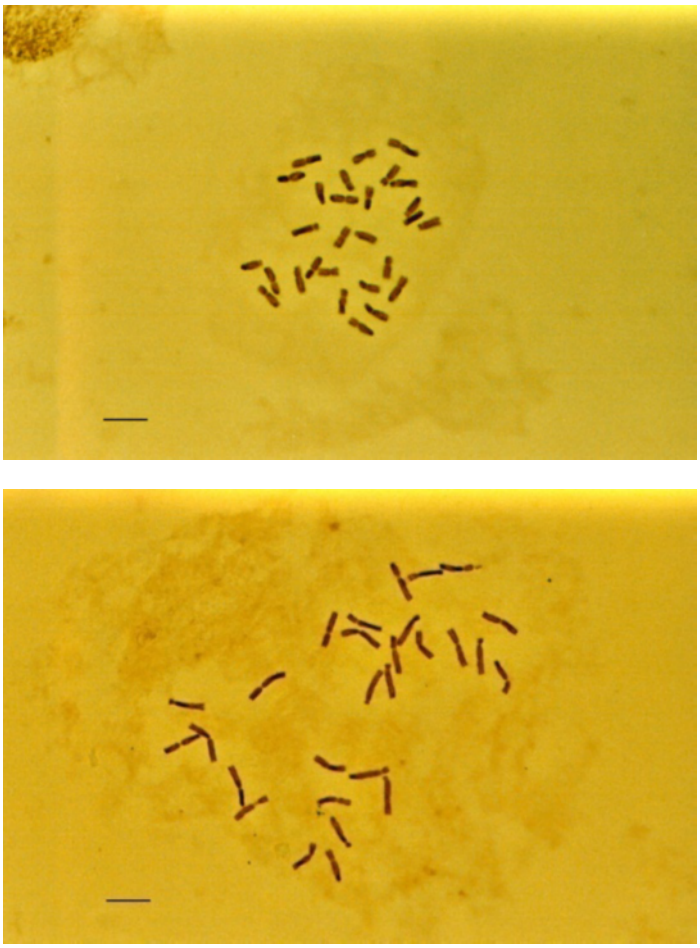

Fig. 4: Top and below figures show accessions of *Aegilops cylindrica* from cluster 1 and 2, respectively (2n=28). Bar represents 10 µm

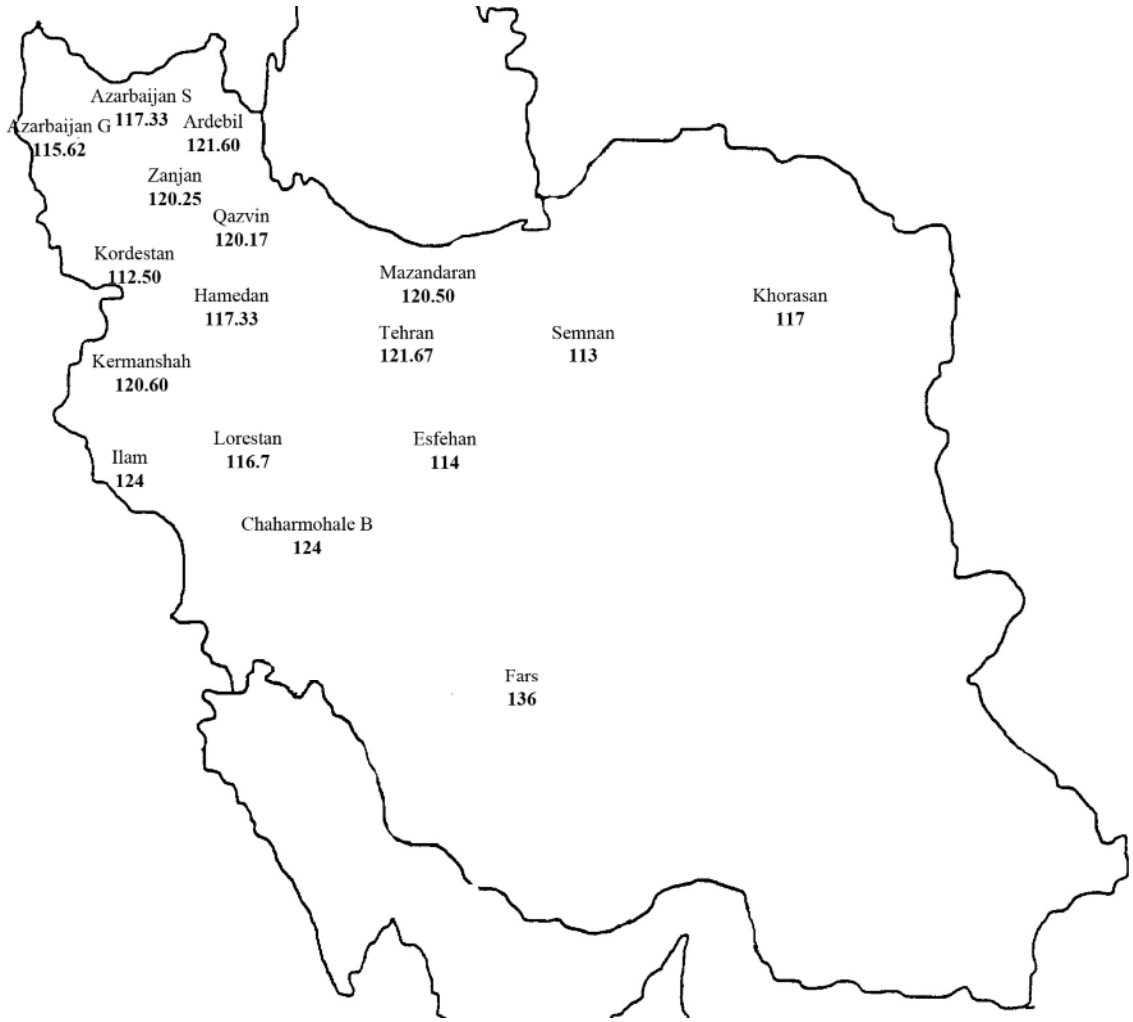


Fig. 5. Variation of DNA peak mode of *Aegilops cylindrica* accessions in different provinces of Iran.

or presence of B chromosomes, but difference on chromosome length was shown in this study. *Cluster analysis of accessions.* Cluster analysis of 100 accessions showed two groups with two different DNA peak modes (fig. 3). The average of DNA peak modes of cluster 1 and 2 was 113.37 and 128.91, respectively. Plants with smaller DNA content may be able to complete the annual growth cycle faster than those with larger genomes (Bennett & Leitch 1995). This specialty of accessions can be beneficial for time of growth in wheat. Also, there was no significant difference in morphological traits of accessions with high and low DNA peak mode. But interestingly, accessions with smaller and larger DNA content had smaller and larger chromosome lengths, respectively (fig. 4).

*Relationship of DNA content and geographical distribution in Iran.* The DNA peak mode of *Ae. cylindrica* in different provinces of Iran proved that there is diversity for DNA content in different areas of Iran (fig. 5). Cluster analysis was used for the classification of provinces of Iran by their average of DNA peak mode. Cluster 1 includes areas around Caspian Sea and West of Iran and average of DNA peak of this cluster was 121.59. Other provinces of Iran that were from Center, North East and North West of Iran were placed in cluster 2 and average of DNA peak of this cluster was 102.87. Interestingly, Fars province of Iran showed the largest DNA peak mode was 136 in average and this province was located alone in cluster 3 (fig. 6). The variation of DNA peak mode among *Ae. cylindrica* accessions is probably due to the gain or loss of DNA content during the evolution of these species and cytotypes in different environments. May be, changes in copy number of certain DNA sequences are responsible for the changes in DNA amount and

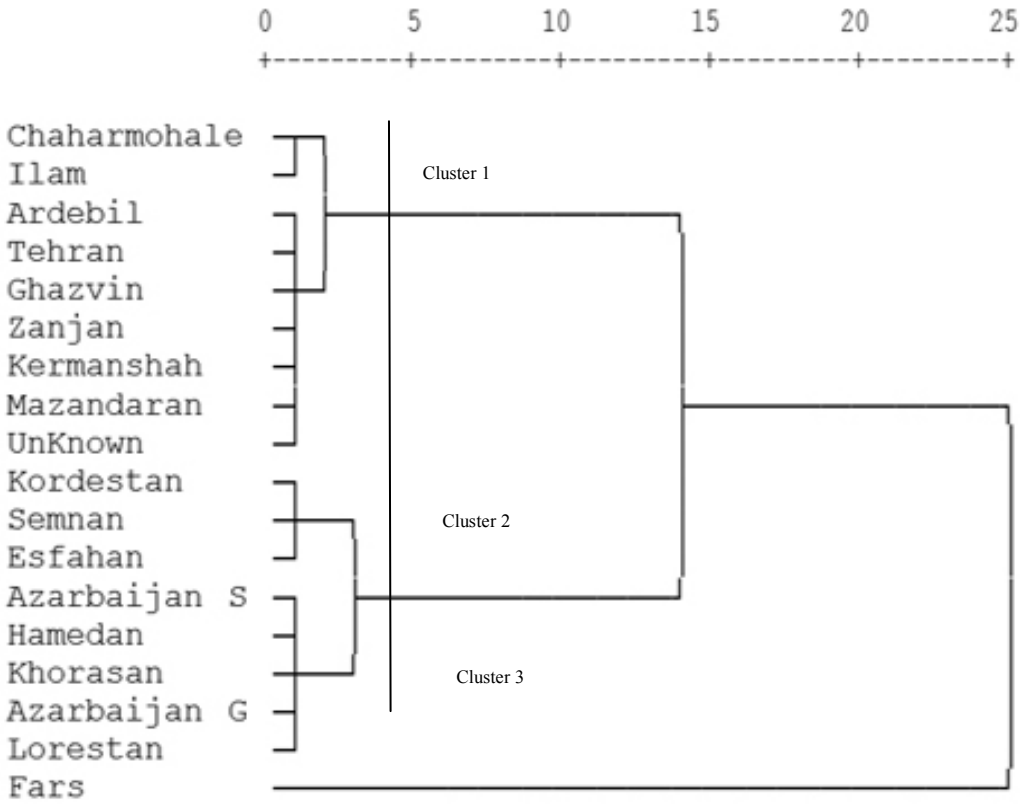


Fig. 6. Cluster analysis of different provinces of Iran by their average of DNA peak mode**.**

probably differences in DNA amount within species of *Ae. cylindrica* are predominantly associated with differences in the amounts of repetitive sequences. It has yet to be clearly demonstrated that amplification of a specific DNA sequence is directly responsible for increase in DNA amount. In addition, differences between DNA peak mode of different accessions of *Ae. cylindrica* estimated by flow cytometry were weakly associated with microclimatic gradient likewise, other research in wild barley (Kalendar *et al*. 2000).

## Conclusion

In genera such as *Aegilops*, consisting of a large number of species, the identification and verification of species based only on morphological traits can be difficult. We propose here that flow cytometric determination of relative nuclear DNA values can be used as a simple and routine method, which can serve as supplementary analysis during identification and maintenance of accessions. Our result proved that, flow cytometry gave a quick and very reliable determination of the ploidy level of *Ae. cylindrica*. Also, this article showed that *Ae. cylindrica* accessions of Iran are tetraploid but there is diversity in DNA content and chromosome length of this species in different areas of Iran and finally, the knowledge about DNA content of *Ae. cylindrica* provides useful information and can be beneficial for breeders for using as a material in their wheat breeding programs.

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