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# DETERMINATION OF PLOIDY LEVELS OF SOME POPULATIONS OF AGROPYRON CRISTATUM (POACEAE) IN IRAN BY FLOW CYTOMETRY *

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**Abstract –** Flow cytometry (FCM) was used to determine the ploidy levels of six different populations of *Agropyron cristatum* (L.) Gaertn. (Poaceae) in Iran, using a diploid cultivated barley (*Hordeum vulgare* L.) as an internal reference. According to flow cytometric data, tetraploid (2n = ± 28) and hexaploid (2n = ± 42) levels were detected among the examined populations and these results were supported by chromosome counting on the same materials included in this study. The mean nuclear DNA content (2C value) of the populations were then estimated, ranging from 26.41 to 27.56 pg for two varieties of *Agropyron cristatum* subsp. *pectinatum,* and 43.47 pg for *Agropyron cristatum* subsp. *incanum.* The relationships between morphological variations observed among the taxa and ploidy levels were also discussed.

**Keywords –** Flow cytometry, ploidy determination, nuclear DNA content, *agropyron cristatum*.

## 1. INTRODUCTION

*Agropyron cristatum* (L.) Gaertner is a xerophytic, Eurasian complex species, which probably originated from central Asia and is indigenous to this area, including parts of the former USSR, China, Afghanistan, Turkey, and Iran [1 and 2]. This economically important grass is widely used on arid rangelands in the United States and Canada [1]. *Agropyron cristatum* complex in Iran is confined primarily, if not exclusively, to the Alborz mountain range, which extends west to east from Turkey to Afghanistan in the northern part of the country [1]. However, Bor [3] in Flora Iranica did not cite any *A. cristatum* collections. He identified most specimens as *A. pectiniforme* Roemer and Schultes, and the few remaining specimens as *A. imbricatum* (Bieb.) Roemer and Schultes.

*Agropyron cristatum* complex exists naturally at three ploidy levels (diploid, 2n=2x=14; tetraploid, 2n=2x=28; and hexaploid, 2n=6x=42) [4, 5]. Dewey and Asay [1] reported all three ploidy levels of the complex from Iran, but refrained from recognizing any subspecific taxon.

Determination of ploidy levels of plants is conventionally conducted by means of chromosome counting, which is time consuming, and in some species rather difficult due to the high number of chromosomes [6]. Alternatively, flow cytometry (FCM) analysis of the nuclear DNA content can be used as an efficient method for rapid detection of ploidy level in plants. This method has been used in several studies including those for plant breeding [7], sex determination in dioecious plants [8],

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cytological study of interspecific hybrids [9], detection of aneuploidy in wheat [10] and screening induced autotetraploidy in the diploid banana [11]. Furthermore, nuclear DNA content (2C value) has been used extensively as an effective tool to estimate the genome size in cultivated and wild species [12-14] and for distinguishing taxa in several groups of angiosperms [15 and 16].

The present study aims to determine the ploidy levels and nuclear DNA content (2C value) of some populations of two *Agropyron cristatum* subspecies and varieties in Iran by FCM, and to compare the results with the earlier reports which were based on chromosome counting [1, 5 and 17].

## 2. MATERIALS AND METHODS

Seeds used in this analysis were collected in Iran by the senior author. Some seeds were also obtained from the Isfahan Shahid Fozveh Center of Seed Technology (Table 1). Nomenclature is the same as that used in the flora of Turkey [18], and the voucher specimens were deposited in the Herbarium of Isfahan University.

For preparation of nuclear samples, young and fresh leaves were obtained from seedlings raised in the greenhouse. Punched discs with a diameter ca. 0.5 cm from fresh leaves were chopped with a sharp razor blade for 30 to 60 sec. in about 400 µl of isolation buffer (the commercial Partec extraction buffer). The suspension was filtered through a 50 µm cell-trics disposable filter and mixed with 1.6 ml staining solution and incubated for 30 to 60 sec. [6 and 12]. A diploid cultivated barley (*Hordeum vulgare* L.; 2n = 14) with known DNA content (2C value) [15] was used as an internal reference [13].

Flow cytometry analysis was performed using the Partec CyStain UV Precise (code No. 05-5002: Partec GmbH, Germany). Analysis of the relative fluorescence intensity of nuclei isolated from young leaf tissue yielded a histogram showing a dominant peak corresponding to nuclei in the G1 phase of the cell cycle and a minor peak corresponding to the G2 phase. To estimate ploidy level, the position of the G1 peak on a histogram was compared to that of *Hordeum vulgare* and the results were presented as DNA index. A software package equipped with the flow cytometer was used to calculate the peak positions and their areas. In addition, the nuclear DNA content (2C value) of each sample in pg DNA was calculated relative to the value for *Hordeum vulgare* L. that had been previously estimated to be 10.40 ± 0.12 pg [15]. Then:

$$\text{2C value of sample i} = \text{DNA index of the sample i} \times 10.40$$

The flow cytometric data were analyzed through an ANOVA and a cluster analysis. For clusting analysis, an unweighted pair-group method using arithmetic averages (UPGMA) and Euclidean coefficient were used. All statistical computations were performed using the STATISTICA version 4.5 (1993) of StatSoft Inc.

Observations were made on root tip cells of the same materials as included in the present investigation, according to Assadi [17], with some modifications. For this purpose, freshly root tips of potted seedlings were treated in a saturated solution of 1-bromonaphthalene for about 4 h at room temperature, fixed in 3:1 ethanol - acetic acid and stained in Schiff´s reagent after hydrolyzing in 1 *M* HCl for 3 min at 60˚C. The preparations were made using the squash method in a drop of 2% acetoorcein. The computer photomicrographs were taken of suitable mitotic cells.

Table 1. The origin of plant materials used in this analysis

| Code | Species | Origin |
|---|---|---|
| A1 | *Agropyron cristatum* subsp. *pectinatum* var. *pectinatum* | West Azarbaijan, Urmie, Shohada valley, 1650 m. |
| A2 | *Agropyron cristatum* subsp. *incanum* | West Azarbaijan, Seru, at the Turkish-Iranian border, Typic hills, 1600 m. |
| A3 | *Agropyron cristatum* subsp. *pectinatum* var. *imbricatum* | Tehran, Chalus Road, Kandavan elevations, 2100-2300 m. |
| A4 | *Agropyron cristatum* subsp. *pectinatum* var. *pectinatum* | Semnan, Shahmirzad, 1800 m. |
| A5 | *Agropyron cristatum* subsp *pectinatum* var. *pectinatum* | Kohkiloye and Boyer Ahmad, 310R (cultivated in Fozveh) |
| A6 | *Agropyron cristatum* subsp. *pectinatum* var. *imbricatum* | Semirom, Hanna, cultivated |

## 3. RESULTS

Flow cytograms derived from this analysis (Fig. 1, A1-A6), showed sharp DNA peaks at channel 50 indicating internal reference. These positions were constant on all cytograms. In contrast, minor DNA peaks representing G1 nuclei from plants of unknown ploidy appeared on channels between 130 to 140 and 215, corresponding to the tetraploid and hexaploid levels, respectively. The results of flow cytometric analysis were summarized in Table 2. Significant variations (F=273295**) are found between the examined populations with respect to the mean and variance of DNA intensity (channel number) of nuclei, detected on the G1 peak of each ploidy level (Table 3).

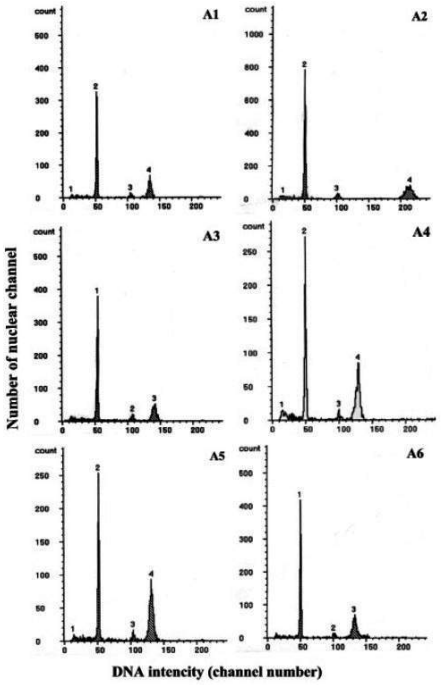


Fig. 1. Flow cytograms of 6 populations of *Agropyron cristatum* (L.) Gaertner in Iran (Table 1), demonstrating genome size invariance between test samples (A1 to A6), and *Hordeum vulgare* L. as an internal reference

Nuclear DNA content (2C values) estimation of the samples were also presented in Table 2. Among examined taxa of *A. cristatum*, 2C values estimated within a range of 26.41 to 43.47 pg. There was a noticeable gap in 2C values separating the taxa into two subgroups.

Table 2. Summarized data of FCM analysis including DNA index*, nuclear DNA content (2C value) and chromosome numbers of 6 different populations of *Agropyron cristatum* subspecies and varieties in Iran. The mean, mode and CV % (coefficient of variations) are related to the DNA intensity (channel number) of G peaks on the flow cytograms (Fig. 1)

| Sample code | Mode | Mean | Area % | CV % | DNA index | 2C value (pg) | Ch. number |
|---|---|---|---|---|---|---|---|
| A1 | 2.596 | 2.578 | 0.422 | 1.159 | 2.578 | 26.83 | 28 |
| A2 | 4.215 | 4.180 | 0.504 | 1.102 | 4.179 | 43.47 | 42 |
| A3 | 2.660 | 2.639 | 0.381 | 0.983 | 2.639 | 27.45 | 28 |
| A4 | 2.580 | 2.548 | 0.765 | 0.900 | 2.583 | 26.83 | 28 |
| A5 | 2.519 | 2.540 | 0.881 | 1.179 | 2.540 | 26.41 | 28 |
| A6 | 2.660 | 2.650 | 0.509 | 1.380 | 2.651 | 27.56 | 28 |

* DNA index = peak position of sample / peak position of reference (Fig. 1)

Table 3. ANOVA for 6 populations of *Agropyron cristatum* were used in this study. The analysis was performed based on the mean and variance of DNA intensity (channel number) of the G1 peaks for each sample, and their appearance on channels between 100 to 250. Data were obtained from flow cytometric analysis (Fig. 1)

| | A1 | A2 | A3 | A4 | A5 | A6 |
|---|---|---|---|---|---|---|
| Total count | 1733 | 4662 | 2096 | 1979 | 1831 | 2272 |
| Mean | 134.68 | 213.14 | 139.42 | 128.23 | 131.26 | 132.67 |
| $S^2x$ | 5.06 | 33.2 | 10.55 | 3.04 | 5.04 | 7.54 |

| Source of variations | SS | df | MS | F |
|---|---|---|---|---|
| Between taxa | 20401473.53 | 5 | 4080294.706 | 273295** |
| Within taxa | 217598.15 | 14569 | 14.93 | |
| Total | 20619071.68 | 14574 | | |

Table 2 was used as data matrix for clustering analysis, including A1 to A6 as OTUs (6 OTUs × 6 characters, excluding the chromosome numbers). As can be seen on the obtained phenogram (Fig. 2), two main clusters are formed, separating *A. cristatum* subsp. *incanum* (A2) from the subsp. *pectinatum*. The first cluster (group 1, Fig. 2), is divided in two subgroups (1.1 and 1.2, Fig. 2), corresponding to the var. *pectinatum* (subgroup 1.1) and var. *imbricatum* (subgroup 1.2).

According to the DNA index (Table 2) and dendrogram obtained from cluster analysis (Fig. 2), the samples can be divided into two major groups: A1, A3, A4, A5 and A6 with a DNA index between 2.54 and 2.65, and as a single representative of group 2, A2 with a DNA index of 4.18. Group 1 can be subdivided in A1, A4, and A5 (1.1), and A3 and A6 (1.2). Members of 1.1 show lower DNA index than members of 1.2, indicating a variation in DNA content, and consequently, in chromosome numbers, probably by ±1 chromosome. A2 shows a 1.6 times higher DNA content as the samples of group 1. This indicates a chromosome number of 42 or more (it is considered that the samples of group 1 possess 2n = 28 chromosomes).

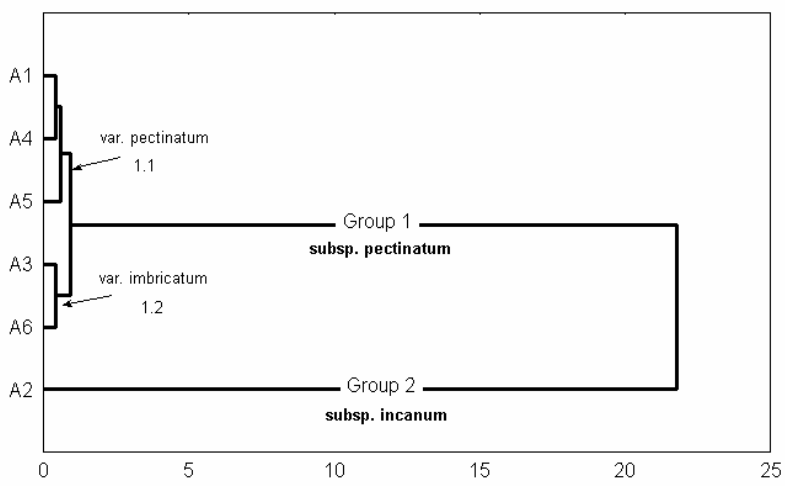


Fig. 2. Cluster analysis of 6 populations of *Agropyron cristatum* subspecies and varieties in Iran based on data obtained from FCM analysis. Data were analyzed using UPGMA and Euclidean coefficient

Photomicrographs of the mitotic root tip cells of the test samples have been shown in Fig. 3 and the chromosome numbers have been presented in Table 2. Among the examined taxa of *A. cristatum*, chromosome numbers observed by a direct counting varied within a range of 28 to 31 in tetraploid populations (subsp. *pectinatum* and its two varieties), and from 35 to 44 in hexaploid population (subsp. *incanum*). The rate of aneuploidy was less (3-4 %) in tetraploids and most of the root cells had 28 chromosomes. In contrast, there was much more aneuploidy (about 18.9 %) in the hexaploid population.

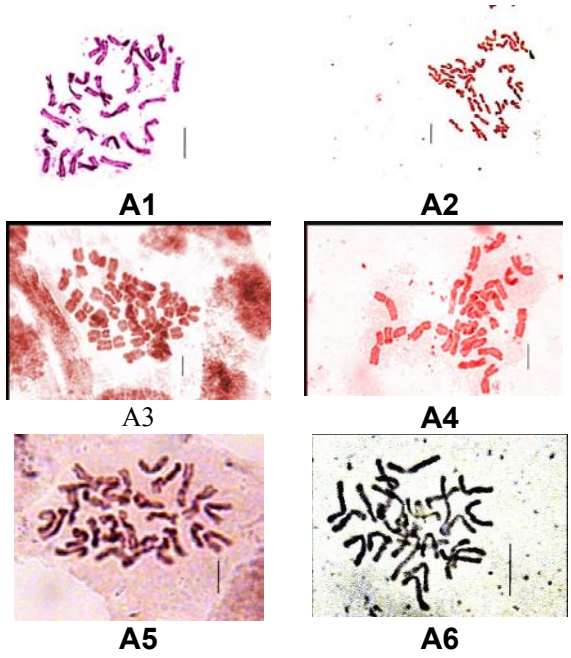


Fig. 3. Photomicrographs showing mitotic root tip cells of 6 populations of *Agropyron cristatum* subspecies and varieties (A1-A6; Table 1): A1) subsp. *pectinatum* var. *pectinatum*, 2n = 28, A2) subsp. *incanum*, 2n = 42, A3) subsp. *pectinatum* var. *imbricatum*, 2n = 28, A4) subsp. *pectinatum* var. *pectinatum*, 2n = 28, A5) subsp. *pectinatum* var. *pectinatum*, 2n = 28 and A6) subsp. *pectinatum* var. *imbricatum* (Bar = 10

## 4. DISCUSSION

Iranian *Agropyron cristatum* complex occurred at three ploidy levels; 2n=14, 28 and 42 [1 and 5]. Tetraploids are the most common form and are found throughout the entire natural habitat of this taxon. Hexaploid populations occurred only in the Azarbaijan province in northwestern Iran. Although Gentry in 1955 collected an accession of diploid *A. cristatum* at a site on the north slope of Mt. Sabalan in the Azarbaijan province [1], it was not present in our collections.

As shown in Table 2, there was one specimen (A2) with DNA index of 4.18 that indicates a hexaploidy level (2n=42). Hexaploids have previously been reported by Dewey and Asay [1] and Assadi [17] from near Maku and Bazargan at the Turkish-Iranian border, almost 300 km from the type location of this specimen. The hexaploid specimen of this study was totally hairy, greish glaucous and coarse, fitting the description of *A. cristatum* subsp. *incanum* described from an adjacent area in Turkey [17, 18]. Other specimens were tetraploid (Fig. 2) with DNA index ranges of 2.54 to 2.65 (Table 2). The DNA index for *A. cristatum* var. *pectinatum* was 2.54 to 2.58 (A1, A4 and A5), whereas for *A. cristatum* var. *imbricatum* it was 2.64 and 2.65 (A3 and A6). There was a small difference between *A. cristatum* var. *pectinatum* and *A. cristatum* var. *imbricatum* with respect to the rate of DNA index and consequently, nuclear DNA content. In fact, the mean of DNA content was 26.26 pg for 3 populations of var. *pectinatum* and 27.50 pg for 2 populations of var. *imbricatum* (Table 2), indicating a difference of 1.24 pg. According to Melderis's treatment of *Agropyron cristatum* in the flora of Turkey [18], the two varieties are separated by only a minor character of glabrous or sparsely pilose spikelets. Small differences in DNA content within *Agropyron* taxa may be due to the presence or absence of satellite chromosomes [14], or due to the aneuploidy observed within the taxa, as mentioned in the results. But the differences in DNA content between the two examined varieties exceeds the probable DNA content of satellite chromosomes, since the average DNA content of an *Agropyron* chromosome has previously been reported as about 1 pg [14]. The satellite chromosomes were not discussed in this study.

Although the ploidy level tends to be associated with morphology [1], morphological variations among Iranian *A. cristatum* subspecies and varieties are not extensive and spike indumentum was the only noticeable morphological difference between the examined populations. The specimens A1, A4 and A5 (Table 1) were glabrous throughout, but two specimens (A3 and A6) had sparsely pilose glumes and lemmas, and only one specimen had densely pubescent spikelets. The results of cluster analysis based on flow cytometric data (Fig. 2) clearly fit with those characteristics mentioned above. However, because of the shortage of clear-cut morphological differences [1, 4], there is no proper explanation for the relationships between ploidy level and observed morphological variations among the examined taxa. To be able to conclude whether the spike indumentum is really correlated with ploidy level or DNA content, more specimens have to be examined, and such a survey has to be made including all known *Agropyron* taxa of various locations.

The nuclear DNA contents (2C value) of the selected specimens which have not been reported previously, were estimated in the present investigation. Vogel et al [14] determined the mean DNA content of 3 accessions of the diploid *Agropyron cristatum* (L.) Gaertner, but they did not analyze the tetraploid and hexaploid strains. Considering the known range of genome size in angiosperms (1C ranging from 0.2 to 127.4 pg) [14, 16], the results of this study, as well as the results from Vogel et al. [14] indicate that the different taxa of *Agropyron cristatum* complex have intermediate to small

genoms. Exact knowledge of genome size is important in many areas of research including genome organization, plant evolution and ecological adaptation of germplasm [16].

Finally, ploidy levels among populations of two subspecies and varieties of *A. cristatum* complex in Iran, observed by FCM, were also supported by the chromosome counting in the present study. These results are comparable with those previously reported by Dewey and Asay [1] and Assadi [17] on the base of chromosome count. Obviously, the most reliable method of determining ploidy level is by counting the number of chromosomes in metaphase. However, the results of this investigation confirmed the usefulness of FCM for the analysis of DNA content as well as ploidy level in *A. cristatum* subspecies and varieties.

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