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DETERMINATION OF NUCLEAR DNA CONTENT AND PLOIDY OF SOME *BROMUS* L. GERMPLASM BY FLOW CYTOMETERY

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Abstract

The objective of this study was to determine nuclear DNA content and ploidy of 48 *Bromus* L. gene bank accessions obtained from USDA by flow cytometry. Based on the results of the flow cytometric analysis, the mean 2C nuclear DNA content of *Bromus* accessions used in the study varied between 11.43 pg with 26.62 pg. The nuclear DNA content differences among *Bromus* L. accessions were statistically significant at $P<0.01$ level. The accessions were easily separated into three distinct groups according to their DNA contents. The mean 2C nuclear DNA content of the groups were 11.82, 22.43 and 26.17 pg (min. and max. values of group were between 11.43–12.65 pg, 21.45–22.77 pg, 25.48–26.62 pg, respectively). Nuclear DNA content of the accessions were correlated with their ploidy levels by counting chromosomes of the one plant from each group with classical staining methods. In conclusion, majority of the *Bromus* accessions (33 out of 48) analysed in the study were tetraploid with 2n=4x=28 chromosomes. Ten and five of the remaining accessions were octaploid (2n=8x=56) and (2n=10x=70) decaploids, respectively. No decaploid *B. inermis* plant reported until today. However, decaploids were reported for *B. riparius* and *B. biebersteini* species. Therefore, decaploid plants determined in this study should be considered as either *B. riparius* or *B. biebersteini*.

Key words: *Bromus* L., Flow cytometry, Nuclear DNA content, Ploidy.

Introduction

Bromus (L.) is one of the most important genus of forage grasses including more than 100 C3 grass species. The genus is widely distributed in Asia, Europe, Africa, and the America (Williams *et al*., 2011). A few perennial species of *Bromus* are widely cultivated for their attributed agricultural characteristics as forage species while few others are very effective in soil conservation. Smooth bromegrass (*Bromus inermis* Leyss.) and meadow bromegrass (*Bromus riparius* Rehm.) are the two most widely cultivated species of genus *Bromus* (Tuna *et al*., 2001). Both the species are members of the *Bromus inermis* complex of section *Pnigma* together with a few other species with similar morphology.

Smooth bromegrass (*Bromus inermis* Leyss.) is adapted to cooler climates, resistant to drought and temperature extremes, and distributed over different elevations (Kostopoulou & Karatassiou, 2016). It grows best on deep, well-drained silt or clay loam but may also establish itself in sandy soils (Turk *et al*., 2015). Smooth bromegrass is generally considered to be a species for hay production because of its uniform leaf arrangement, upright tillering and high dry matter yield while it is also valuable as pasture and silage (Biligetu & Coulman, 2010). It is a deep-rooted grass that spreads extensively via underground rhizomes that forms dense stands (Casler & Carlson, 1995). Because of its highly developed root system, smooth bromegrass is resistant to temperature extremes and drought (Gould & Shaw, 1983; Serin & Tan, 2009; Turk *et al*., 2015). At the same time, it is one of the most freezing-tolerant perennial grass species (Williams *et al*., 2011).

Meadow bromegrass, *Bromus riparius* Rehm. (also called *B. erectus* Huds. and *B. biebersteinii* Holub) is native to southeastern Europe, the Caucasus, Turkey, and central Asia (Knowles *et al*., 1993). It is a reduced creeping type of bromegrasses with good ability for regrowth. It has more uniform seasonal production than smooth bromegrass and therefore is generally considered to be a species for grazing. Meadow bromegrass is also start growing a few weeks earliear in spring comparing to smooth bromegrass and resistant to brown leaf-spot *Pyrenophora bromi* Died., which causes severe losses in smooth bromegrass.

The species included in *Bromus inermis* complex have varying ploidy levels from diploid to decaploid (Carnahan & Hill, 1960; Armstrong 1987; Vogel *et al*., 1996). Ploidy is an essential information for identification and classification of genetic resources of a crop species. The information is also needed to determine the best strategy to use the genetic resources in research and breeding programmes. Otherwise, it causes waste of limited sources of the breeders such as time, energy and money.

Traditionally, ploidy analysis is done by counting mitotic chromosomes of cells of stained root tip tissues with a light microscope. But, the method is long, laborious, need expertise and often difficult with species which have small chromosomes and high ploidy levels, and can lead to misclassification germplasms (Brummer *et al*., 1999). The chromosomes are made of mostly DNA that are tightly coiled around positively charged proteins called histones, and there is a positive correlation between nuclear DNA amount and ploidy level. This close relation makes nuclear DNA content to be used as an estimate of ploidy level. Nuclear DNA content is also a useful information for botanical, genetical, and evolutionary studies. Flow cytometry has become the choice of the method in nuclear DNA content anaylsis (Jaskani *et al*., 2005) due to its easiness, accuracy and relatively low cost.

Recently, we have obtained 48 accessions of bromegrasses from USDA with only the species name on the package to use in our bromegrass breeding programme. The objectives of this study are to determine the nuclear DNA content and ploidy levels among 48 accessions by flow cytometery.

Materials and Methods

Plant materials: In this study, 48 *Bromus* L. accessions obtained from USDA were used as plant material. Forty-five of these accessions were originated from the People's Republic of China, the United States of America (USA), Russia, Mongolia and Kazakhstan. The origin of the remaining three populations were unknown. Complete list of accessions is given in Table 1.

Germination and growing: The seeds were germinated between two filter papers placed into a dispossible petri dish. Captain solution (Captain WP 50%, 250 g/100 lt) was used as moisturizing agent in germination. Ten seedlings were transplanted into plastic pots (7x7x7) after germination, and the seedlings were grown in greenhouse until they were analysed by flow cytometery.

Nuclear DNA content analysis: Nuclear DNA content analysis was carried out in the Plant Genetic and Cytogenetics Laboratory of Field Crops Department of Agricultural Faculty of Tekirdag Namik Kemal University, Tekirdag/Turkey using a flow cytometery. Three individual plants were analysed for each accessions and accession mean was calculated. Fresh leaf tissues from approximatelly 6 weeks old, healthy plants were used in the analysis. The samples were prepared by using a commercial kit, the 'CyStain PI absolute P' nuclei extraction and staining kit (Partec GmbH, Munster) according to the manufacturer's instructions. Samples were analysed using a Partec CyFlow Space flow cytometery (Munster, Germany). The absolute DNA content of sample plants was calculated based on the ratios of the G1 peak means of sample and internal standard. Barley (Sladoran) was used as internal standard.

An ANOVA was performed on nuclear DNA content data by using the SPSS Ver. 15.0 software for Microsoft Windows. Experimental design was a randomly complete parcel design with 3 replications. The differences between the mean values were compared by Duncan's multiple range test ($p \leq 1$).

Preparations and chromosome counts: The preparations were made for only 3 plants (one for each ploidy group) according to the protocol described in detail by Hasterok *et al*., (2006). Briefly, dehusked seeds were germinated between two filter papers moistened with tap water at 20–22^{o}C in the dark for 3–4 days. Roots grown about 1.5 cm were harvested and immersed immediatelly in ice-cold water for approximatelly 20 h. Roots were fixed in 3:1 (v/v) ethanol/glacial acetic acid at room temperature for one day, and then stored at 4^{o}C until use. Before making preparations, the roots were washed in 0.01 M citric acid–sodium citrate buffer (pH = 4.8) for 20 min and enzymatically digested in a mixture comprising 20% (v/v) pectinase (Sigma, St Louis, MO, USA), 1% (w/v) cellulase (Calbiochem, San Diego, CA, USA) and 1% (w/v) cellulase 'Onozuka R-10' (Serva, Heidelberg, Germany) for 2 h at 37^{o}C. Meristems were dissected out from root tips, squashed in drops of 45% acetic acid and frozen. After freezing, cover-slips were removed and the preparations were dehydrated in absolute ethanol and air dried. A glass coverslip (24×24 mm) was placed over the specimen on the slide after applying 7-10 µl of Vectashield-DAPI mounting-staining medium. It was pressed between filter paper to squeeze out excess mounting medium. Choromosomes of the cells with complete chromosome number were counted by using fluorescent microscope and their images were captured by using a CCD digital camera (Spot RT) attached to microscope.

Results and Discussion

2C Nuclear DNA content of the bromegrass plants were determined successfuly. Low standard deviation values indicating a high degree of precision for the flow cytometry procedure were used in the study (Table 1).

Based on the results obtained in the study, nuclear DNA content of *Bromus* accessions varied between 11.43 and 26.62 pg. The differences among nuclear DNA content of accessions were statistically significant (p<0.01) (Table 1).

The mean nuclear DNA content of bromegrass accessions clearly formed three distinct groups in the study. The mean nuclear DNA content varied from 11.43 to 12.65 pg / 2C in the lowest group, while the mean nuclear DNA content varied from 25.48 to 26.62 pg / 2C in the highest group. In the middle group, the mean nuclear DNA content varied between 21.45 and 22.77 pg / 2C. The flow histograms of one represantative plant for each of the groups are presented below (Fig. 1A-C).

DNA content of the plants were correlated to their ploidy levels by counting mitotic chromosomes of one plant from each group. Cytogenetical analysis showed that representative plants of groups with lowest, medium and highest DNA content had 2n=28, 56 and 70 chromosomes, respectively. Since the number of basic chromosomes in genus *Bromus* L. is x = 7 (Sheidai *et al*., 2008; Williams *et al*., 2011; Tuna 2000), it is accepted that plants with 2n = 28 chromosomes are tetraploids, 2n = 56 chromosomes are octaploids, 2n = 70 chromosomes number are decaploid. The images of mitotic chromosomes for each ploidy levels are presented in Fig. 2.

These results indicated that 33 of the *Bromus* accessions analysed in the study were tetraploid with 2n=4x=28 chromosomes. The number of the octaploid (2n=8x=56) and (2n=10x=70) decaploid accessions were 10 and 5 respectively. Nuclear DNA content in bromegrass accessions were higly and positively correlated with ploidy level.

The results of nuclear DNA content analysis carried out in the current study are very similar with the previous results reported by Tuna *et al*., (2001). In their study, Tuna *et al*., (2001) reported mean 2C nuclear DNA content of tetraploid and octaploid *B. inermis ssp. inermis*, as 11.74 and 22.28 pg, respectively. They didn't found any decaploid *B. inermis* in their study. No decaploid *B. inermis* plant is reported until today. However, decaploids were reported for *B. riparius* and *B. biebersteini* species (Armstrong 1987; Vogel *et al*., 1996; Tuna *et al*., 2001). Therefore, decaploid plants determined in this study should be considered as either *B. riparius* or *B. biebersteini*.

Table 1. Mean 2C nuclear DNA contents (pg), standard deviation, ploidy levels, origin of country and serial number of *Bromus* L. accessions used in the study.

Accession number	Species	Origin	Nuclear DNA content (pg)	Standard deviation	Ploidy levels
PI 655131	*Bromus inermis* subsp. inermis	Mongolia	26.62 a*	0.250	Decaploid
PI 628278	*Bromus inermis* subsp. inermis	Unknown	26.43 a	0.080	Decaploid
PI 619019	*Bromus inermis* subsp. inermis	Mongolia	26.06 b	0.055	Decaploid
PI 598572	*Bromus inermis* subsp. inermis	Russia	25.96 b	0.040	Decaploid
PI 598577	*Bromus inermis* subsp. inermis	Russia	25.48 c	0.235	Decaploid
PI 642845	*Bromus inermis* subsp. inermis	USA	22.77 d	0.075	Octaploid
PI 636579	*Bromus inermis* subsp. inermis	China	22.61 d	0.205	Octaploid
PI 642844	*Bromus inermis* subsp. inermis	USA	22.53 de	0.110	Octaploid
PI 21403	*Bromus inermis* subsp. inermis	Mongolia	22.29 ef	0.530	Octaploid
PI 25093	*Bromus inermis* subsp. inermis	Kazakhstan	22.08 fg	0.460	Octaploid
PI 598570	*Bromus inermis* subsp. inermis	Russia	22.07 fg	0.195	Octaploid
PI 584449	*Bromus inermis* subsp. inermis	USA	22.05 fg	0.405	Octaploid
PI 655218	*Bromus inermis* subsp. inermis	USA	21.91 g	0.185	Octaploid
PI 619004	*Bromus inermis* subsp. inermis	Unknown	21.61 h	0.300	Octaploid
PI 655222	*Bromus inermis* subsp. inermis	USA	21.45 h	0.485	Octaploid
PI 19685	*Bromus inermis* subsp. inermis	Mongolia	12.65 ı	0.050	Tetraploid
PI 13055	*Bromus inermis* subsp. inermis	China	12.19 j	0.190	Tetraploid
PI 610869	*Bromus inermis* subsp. inermis	Mongolia	12.04 jk	0.040	Tetraploid
PI 655721	*Bromus inermis* subsp. inermis	Mongolia	11.97 jkl	0.145	Tetraploid
PI 13111	*Bromus inermis* subsp. inermis	China	11.97 jkl	0.205	Tetraploid
PI 598592	*Bromus inermis* subsp. inermis	Kazakhstan	11.96 jkl	0.065	Tetraploid
PI 18154	*Bromus inermis* subsp. inermis	Mongolia	11.90 j-m	0.050	Tetraploid
PI 12972	*Bromus inermis* subsp. inermis	China	11.90 j-m	0.075	Tetraploid
PI 18223	*Bromus inermis* subsp. inermis	Mongolia	11.88 j-n	0.025	Tetraploid
PI 636583	*Bromus inermis* subsp. inermis	China	11.88 j-n	0.060	Tetraploid
PI 636614	*Bromus inermis* subsp. inermis	Mongolia	11.88 j-n	0.035	Tetraploid
PI 610855	*Bromus inermis* subsp. inermis	Mongolia	11.86 j-n	0.200	Tetraploid
PI 12936	*Bromus inermis* subsp. inermis	China	11.86 j-n	0.064	Tetraploid
PI 632460	*Bromus inermis* subsp. inermis	China	11.86 j-n	0.020	Tetraploid
PI 598581	*Bromus inermis* subsp. inermis	Kazakhstan	11.82 k-n	0.035	Tetraploid
PI 19648	*Bromus inermis* subsp. inermis	Mongolia	11.79 k-o	0.065	Tetraploid
PI 636615	*Bromus inermis* subsp. inermis	Mongolia	11.79 k-o	0.175	Tetraploid
PI 598556	*Bromus inermis* subsp. inermis	China	11.73 k-o	0.080	Tetraploid
PI 598586	*Bromus inermis* subsp. inermis	Kazakhstan	11.71 k-o	0.015	Tetraploid
PI 618974	*Bromus inermis* subsp. inermis	Mongolia	11.70 k-o	0.125	Tetraploid
PI 598585	*Bromus inermis* subsp. inermis	Kazakhstan	11.66 l-o	0.115	Tetraploid
PI 598579	*Bromus inermis* subsp. inermis	Kazakhstan	11.63 l-o	0.060	Tetraploid
PI 636578	*Bromus inermis* subsp. inermis	Unknown	11.63 l-o	0.060	Tetraploid
PI 636576	*Bromus inermis* subsp. inermis	Mongolia	11.63 l-o	0.040	Tetraploid
PI 610865	*Bromus inermis* subsp. inermis	Mongolia	11.60 mno	0.100	Tetraploid
PI 598538	*Bromus inermis* subsp. inermis	China	11.57 mno	0.230	Tetraploid
PI 610847	*Bromus inermis* subsp. inermis	Mongolia	11.56 mno	0.025	Tetraploid
PI 598588	*Bromus inermis* subsp. inermis	Kazakhstan	11.55 mno	0.070	Tetraploid
PI 618991	*Bromus inermis* subsp. inermis	China	11.52 no	0.121	Tetraploid
PI 610882	*Bromus inermis* subsp. inermis	Mongolia	11.52 no	0.010	Tetraploid
PI 632560	*Bromus inermis* subsp. inermis	China	11.45 o	0.000	Tetraploid
PI 636580	*Bromus inermis* subsp. inermis	China	11.44 o	0.075	Tetraploid
PI 598583	*Bromus inermis* subsp. inermis	Kazakhstan	11.43 o	0.015	Tetraploid

*Values with insignificant difference for column are indicated with same letter

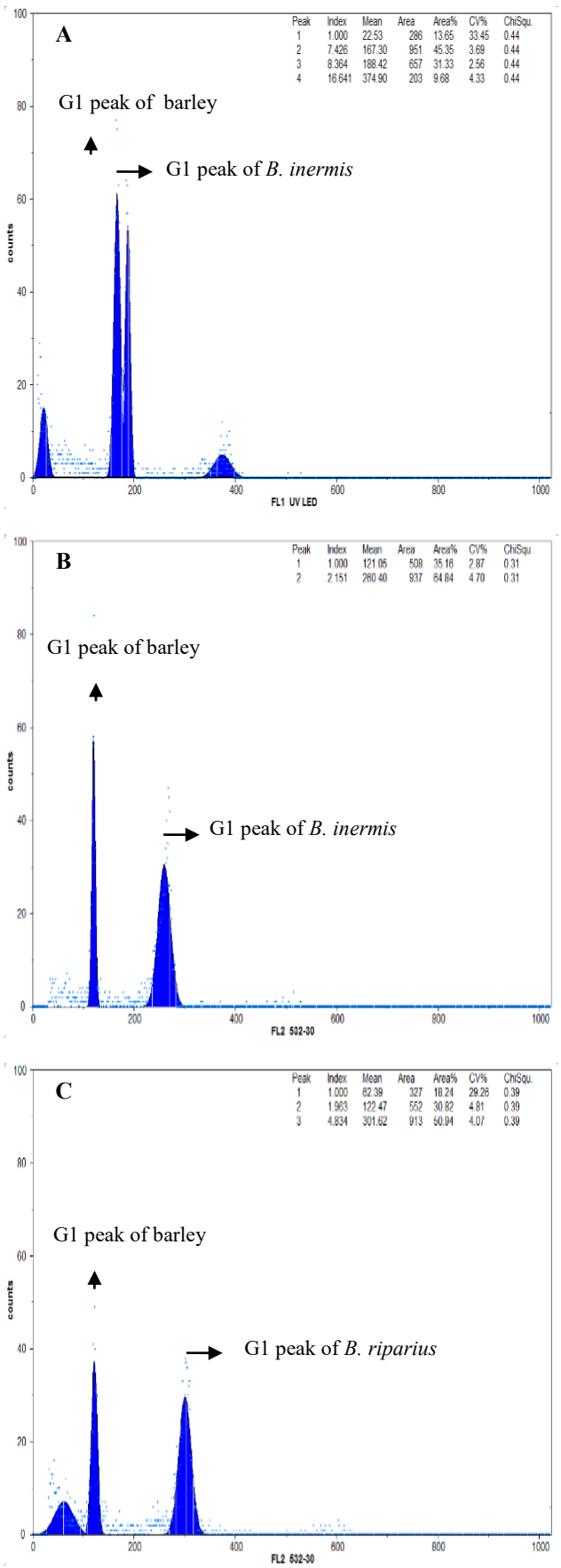

Fig. 1. Flow cytometry histograms, A) G1 peaks of Barley (standard) and tetraploid *Bromus inermis* L., B) G1 peaks of Barley (standard) and octaploid *Bromus inermis* L., C) G1 peaks of Barley (standard) and decaploid *Bromus riparius* L.

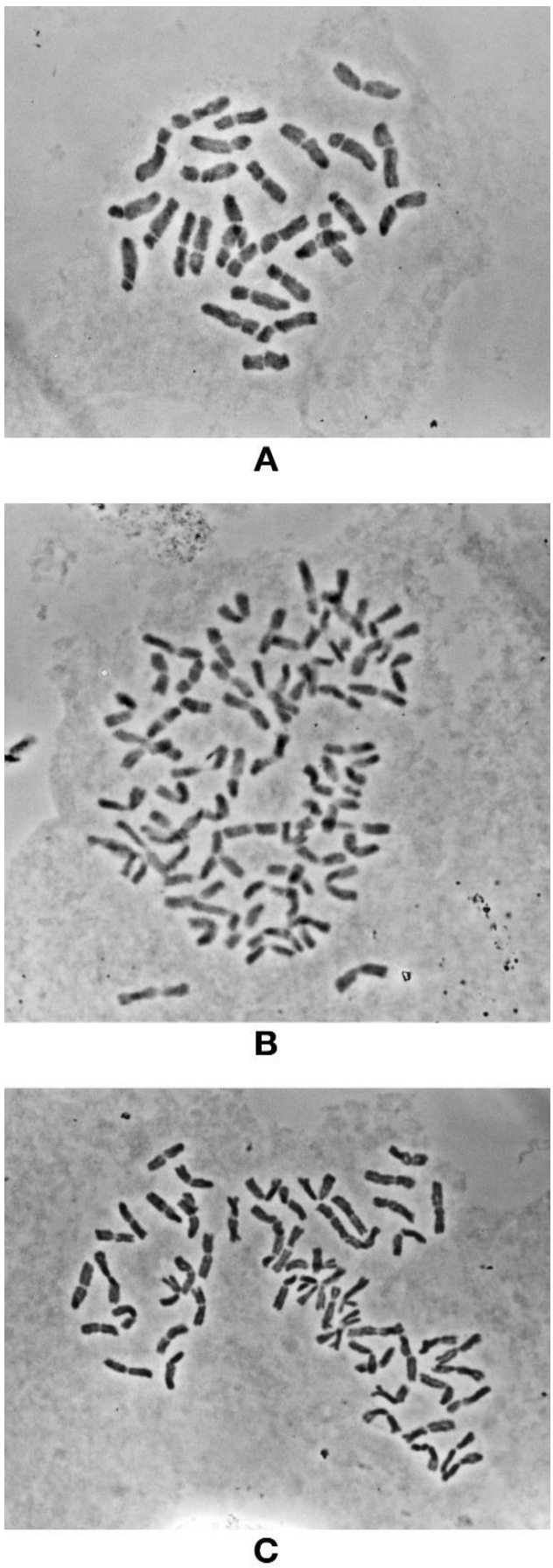

Fig. 2. Somatic chromosomes A) Tetraploid *Bromus inermis* L. (2n=4x=28), B) Octaploid *Bromus inermis* L. (2n=8x=56), C) Decaploid *Bromus riparius* L. (2n=10x=70).

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