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DNA Content and Ploidy Determination of Bromegrass Germplasm Accessions by Flow Cytometry

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ABSTRACT

Species of the genus *Bromus* represent ploidy states from diploid to decaploid. Ploidy determination of *Bromus* germplasm is necessary before it can be effectively used in breeding or genetic studies. The objective of this study was to characterize the ploidy of 322 accessions of four *Bromus* species [*Bromus inermis* Leyss, *B. riparius* Rehm, *B. biebersteinii* Roem and Schult., and *B. inermis* ssp. *pumpellianus* (Scribn) Wagnon] that are in the USDA National Plant Germplasm System (NPGS). Flow cytometry was used to determine DNA content of 10 plants of each accession. Mean DNA contents were correlated to ploidy level with root tip chromosome counts on selected accessions whose DNA content indicated that they represented different ploidy levels. On the basis of DNA content (pg $2C^{-1}$ = DNA content of a diploid somatic nucleus) and chromosome counts, mean DNA content and chromosome number was 22.62 pg $2C^{-1}$ for octaploid *B. biebersteinii* ($2n = 8x = 56$), 26.07 pg $2C^{-1}$ for decaploid *B. biebersteinii* ($2n = 10x = 70$), 11.74 pg $2C^{-1}$ for tetraploid *B. inermis* ($2n = 4x = 28$), 22.28 pg $2C^{-1}$ for octaploid *B. inermis* ($2n = 8x = 56$), 22.72 pg $2C^{-1}$ for octaploid *B. inermis* ssp. *pumpellianus* ($2n = 8x = 56$), 26.5 pg $2C^{-1}$ for decaploid *B. inermis* ssp. *pumpellianus* ($2n = 10x = 70$), 6.14 pg $2C^{-1}$ for diploid *B. riparius* ($2n = 2x = 14$), 22.15 pg $2C^{-1}$ for octaploid *B. riparius* ($2n = 8x = 56$), and 26.64 pg $2C^{-1}$ for decaploid *B. riparius* ($2n = 10x = 70$). Standard deviations of the mean values were 0.88 pg $2C^{-1}$ or less. Most *B. inermis* and *B. inermis* ssp. *pumpellianus* accessions were octaploid (93.75%), while the majority of the *B. riparius* and *B. biebersteinii* were decaploid (92.30%). The *B. inermis* and related species in the USDA NPGS were collected primarily from areas in the former USSR. The NPGS bromegrass germplasm could be enhanced by collections from western and central Europe, the Middle East, and China.

The genus *Bromus* L. contains more than 100 species distributed over all continents (Gould and Shaw, 1983). Smooth bromegrass (*B. inermis* Leyss.) and meadow bromegrass (*B. riparius* Rehm.) are the two most widely used species of the *Bromus* genus in North America. The species name *B. biebersteinii* Roem. and Schultz has been incorrectly applied to meadow bromegrasses in North America until recently (Vogel et al., 1996). Smooth bromegrass and pumpelly brome [*B. inermis* spp. *pumpellianus* (Scribn.) Wagnon.] are closely related since they are completely interfertile and have regular chromosome pairing at meiosis (Armstrong, 1987). The chloroplast restriction patterns for smooth bromegrass, meadow bromegrass, and pumpelly bromegrass are identical (Pilay and Hilu, 1990).

A ploidy series exists within these species which have the base chromosome number of $n = 7$. Reported chromosome numbers for smooth bromegrass are $2n = 28$, 42, and 56 and for meadow bromegrass are 14, 56, and 70 (Tsvelev, 1984; Hill and Myers, 1948; Carnahan and Hill, 1960; Armstrong, 1987; Vogel et al., 1996). The commonly grown form of smooth bromegrass is an autoallooctaploid with a chromosome number of $2n = 8x = 56$, while the tetraploid ($2n = 4x = 28$) is an allotetraploid (Armstrong, 1973; Elliot and Wilsie, 1948; Hill and Meyers, 1948; Carnahan and Hill, 1960; Vogel et al., 1996). Cultivated meadow bromegrass is decaploid with a chromosome number of $2n = 10x = 70$ (Knowles et al., 1993). Meadow bromegrass ($2n = 70$) probably contains the same basic genomes as smooth bromegrass ($2n = 56$) plus a third additional genome (Schultz-Schaeffer, 1960).

Ploidy determinations have traditionally been done by counting chromosomes of stained root tips, but this method is laborious and often difficult with species which have small chromosomes and high ploidy levels and can lead to misclassified germplasm (Brummer et al., 1999). All chromosomes are located in the cell nucleus of plants enabling nuclear DNA content to be used as an estimate of ploidy level. Nuclear DNA content in plants was previously determined by feulgen microspectrophotometry of root tip or shoot tip mitotic cells (Bennett and Smith, 1976). In recent years, flow cytometry has become the preferred technique for estimating the nuclear DNA content because of its ease, quickness, and accuracy (Rayburn et al., 1989; Heslop-Harrison, 1995).

Arumuganathan and Earle (1991a) determined nuclear DNA contents of more than 100 major crop plant species using flow cytometry. Vogel et al. (1999) used flow cytometry to determine the base DNA content of the genomes in the perennial Triticeae. Flow cytometry also has been used to determine the ploidy level of switchgrass (*Panicum virgatum* L.) (Hultquist et al., 1997; Lu et al., 1998), alfalfa (*Medicago sativa* L.) (Brummer et al., 1999); and 13 turfgrass species (Arumuganathan et al., 1999). The amount of DNA in plant cells is expressed in picograms (pg) as a "C" value. (Bennett and Smith, 1976). The letter C stands for a "constant" or the amount of DNA in a haploid nucleus or genome; 2C values represent the DNA content of a diploid somatic nucleus. DNA amounts in picograms

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Abbreviations: NPGS, National Plant Germplasm System; pg $2C^{-1}$, DNA content of a diploid somatic nucleus in picograms; C-value is the DNA amount in the unreplicated haploid nucleus of an organism and stands for "constant."

can be converted to megabase pairs (Mbp) by means of the conversion factor of 1 pg = 980 Mbp (Bennett et al., 2000). Bennett and Smith (1976) reported DNA content values for octaploid *B. inermis* and *B. erectus* Huds. of 23.6 pg and 23.3 pg $2C^{-1}$, respectively, which were obtained with Feulgen microdensitormetry.

The USDA Plant Germplasm System contains over 255 accessions of *B. inermis*, 49 accessions of *B. riparius*, nine accessions of *B. inermis* ssp. *pumpellianus*, and nine accessions of *B. biebersteinii*. The ploidy level of most of these accessions was unknown prior to the completion of this research. Lack of information on ploidy levels limits the utility of germplasm forage breeding programs. Hybridization of plants with different ploidy levels can result in nonviable progeny or genetically unstable progeny (Vogel and Pedersen, 1993).

The objectives of this study were to determine the nuclear DNA content of more than 322 bromegrass accessions in the USDA National Plant Germplasm System that are classified as *B. inermis*, *B. inermis* ssp. *pumpellianus*, *B. riparius*, and *B. biebersteinii*, correlate DNA content with ploidy level for these species, and classify the accessions for ploidy on the basis of DNA content. The ploidy level information was then used to characterize the genomic structure of the smooth, meadow, and pumpelly bromegrass collections.

MATERIALS AND METHODS

Accessions of *B. inermis*, *B. riparius*, *B. biebersteinii*, and *B. inermis* ssp. *pumpellianus* were obtained from the USDA Regional Plant Introduction Station, Pullman, WA, in December 1995. Twenty seedlings of each of 322 accessions (255 *B. inermis*, 49 *B. riparius*, nine *B. biebersteinii*, and nine *B. inermis* ssp. *pumpellianus*) were grown in individual plastic conetainers (22 cm deep, 4 cm in diameter) which contained a mixture of 2:1:1 soil/peat/vermiculite in the USDA Forage Research Lab greenhouse at Lincoln, NE. Plants were maintained in the vegetative condition by repeated clippings.

The procedures described by Arumuganathan and Earle (1991b) were used to determine DNA content per nucleus. Briefly, the procedure consists of preparing suspensions of intact nuclei by chopping plant tissues and lysing protoplasts in a $MgSO_4$ buffer mixed with DNA standards and staining the nuclei with propidium iodide (PI) in a solution containing DNase-free RNase. Fluorescence intensities of the stained nuclei were measured by flow cytometry. Values for nuclear DNA content were estimated by comparing fluorescence intensities of the nuclei of the test population with those of a diploid barley (*Hordeum vulgare* L. cv. Hitchcock) or hexaploid wheat (*Triticum aestivum* L. cv. Arapahoe) internal DNA standard that was included with the tissue being tested.

Approximately 50 mg of fresh, green tissue from a collared leaf of a *Bromus* seedling was excised and placed on ice in a sterile 35- by 10-mm plastic petri dish. About 20 mg of leaf tissue from seedling barley or wheat leaves were added as a standard. 2C complements of DNA per nucleus for the barley and wheat are 10.68 and 34.68 pg, respectively. Barley ($2n = 2x = 14$) and wheat ($2n = 6x = 42$) were used as standards because of the large range in DNA content of the strains analyzed. The leaf tissue (bromegrass and standard) was chopped into 0.25–1.0 mm segments in 1 mL of solution A [24 mL $MgSO_4$ buffer (ice-cold); 25 mg dithiothreitol; 500 μL propidium iodide stock (5.0 mg propidium iodide in 1.0 mL double distilled H_2O); 625 μL Triton X-100 stock (1.0 g Triton X-100 in 10 mL ddH_2O)]. The homogenate was filtered through a 33-μm nylon mesh into a microcentrifuge tube and centrifuged (VS-15 microcentrifuge, Shelton Scientific, Shelton, CT) at 13 000 RPM for 20 s. The supernatant was discarded, the pellet was resuspended in 400 μL of solution B [7.5 mL solution A; 17.5 μL RNase (DNase free)] and it was incubated for 15 min at 37°C before flow cytometric analysis.

The prepared material was analyzed in the University of Nebraska Flow Cytometry Core Research Facilities on a standard FACScan model flow cytometer (Becton Dickinson Immunocytometry system, San Jose, CA). For measurement, PI fluorescence area signals (FL2-A) from 1000 nuclei were

Table 1. Nuclear DNA content of *Bromus* accessions with known chromosome numbers and ploidy level of accessions determined by nuclear DNA content.

Accessions with known chromosome numbers				Accessions with ploidy level determined by DNA content				Cytogenetic analysis
Number of accessions	Chromosome number ($2n$)	DNA pg/2C Mean	SD	Accession number	Ploidy level	DNA pg/2C Mean†	DNA pg/2C SD	PI (s)§ or comments
Bromus biebersteinii								
1§	56	22.62	0.70	1	8*x*	22.62	0.70	PI 325226
2§	70	27.13	1.01	7	10*x*	26.07	0.79	PI 172394, PI 341222
				1	mixed	13.56	1.66	Tetra-, aneuploid DNA content
Bromus inermis* spp. *inermis								
6‡§	28	11.73	0.10	14	4*x*	11.74	0.16	PI 315385, PI 440201, PI 440202, PI 440203, PI 440204, PI 499401
19‡§	56	22.11	0.19	233	8*x*	22.28	0.42	PI 251861, PI 574512, PI 574514, PI 578551
				8	mixed	21.08	3.27	Tetra-, octa- & aneuploid DNA content
Bromus inermis* spp. *pumpellianus								
2§	56	23.09	0.03	7	8*x*	22.72	0.88	PI 372671, PI 562648
				2	10*x*	26.5	0.14	
Bromus riparius								
1‡§	14	6.14	0.09	1	2*x*	6.14	0.09	PI 440215
1§	56	22.76	0.42	2	8*x*	22.15	0.86	PI 315380
11‡§	70	26.53	0.43	41	10*x*	26.64	0.52	PI 440214, PI 536012, PI 536013
				5	mixed	24.65	1.31	Octa-, deca-, & aneuploid DNA content

† Based on all accessions within species and ploidy level for which accession nuclear DNA content standard deviation (SD) for the 10 plants analyzed per accession was less than 1.0 pg.
‡ Chromosome numbers include data on accessions from Armstrong (1987).
§ Chromosome numbers determined or verified in this study on indicated PI accessions listed in the Cytogenetic analysis column.

collected by CellQuest software (Becton Dickinson Immunocytometry system, San Jose, CA). A live gate instrument configuration was used by employing the FL2-2 and FSC parameters which allowed the fluorescence measurement from nuclei to be used to generate a histogram of FL2-A. Mean position of G0/G1 (nuclei) peak of sample and internal standard were determined by analyzing the data by CellQuest software. The mean DNA content per plant was based on the 1000 scanned nuclei. The formula used for converting florescence values to DNA content was: Nuclear DNA content = (mean position of unknown peak)/(mean position of known) × DNA content of known standard.

Ten seedlings were analyzed for DNA content per accession. One seedling per accession was analyzed twice (subsample a and b) to obtain an estimate of laboratory precision which was 0.03 pg for this study. Plants were reanalyzed for nuclear DNA content if the subsample standard deviation for an accession was greater than 1.0 pg, if variation in DNA content among plants indicated that there were differences among plants for ploidy level in that accession, or if a plant had a DNA content that was intermediate between the expected ploidy levels for that species.

After DNA levels were determined, one to six representative accessions (Table 1) with different DNA levels were selected for each species and used for chromosome counting. Cytological investigations were done on root tips. For this purpose, the terminal 1 cm of the end of fresh roots was excised from plants growing in pots and placed in a vial containing 0.05% colchicine. Colchicine was replaced with ethanol:glacial acetic acid (3:1, v/v) after 3 h. For mitotic analysis, root tips were stained with 1% acetocarmine for 1 to 3 h and squashed in a drop of acetic acid. Cells were observed under a light microscope to determine the chromosome number. Approximately 10 cells at metaphase I from a minimum of three plants from each accession selected for chromosome number analyses were observed to determine the chromosome number. We were not able to count chromosome numbers of the decaploid *B. inermis* ssp. *pumpellianus* accessions since root tips with dividing cells were not found.

RESULTS AND DISCUSSION

Accurate determination of the chromosome number is difficult in bromegrasses because of their small chromosome size and high number of chromosomes (Hill and Myers, 1948; Barnett, 1955). Colchicine treatment of the root tips resulted in straighter and smaller chromosomes than the cold water treatment and gave better chromosome spreads which allowed us to make more accurate chromosome counts (Fig. 1, 2, 3, 4). All diploid plants had 14 chromosomes and no aneuploids were observed in the diploid accession. Only two aneuploid plants were observed among all tetraploid accessions

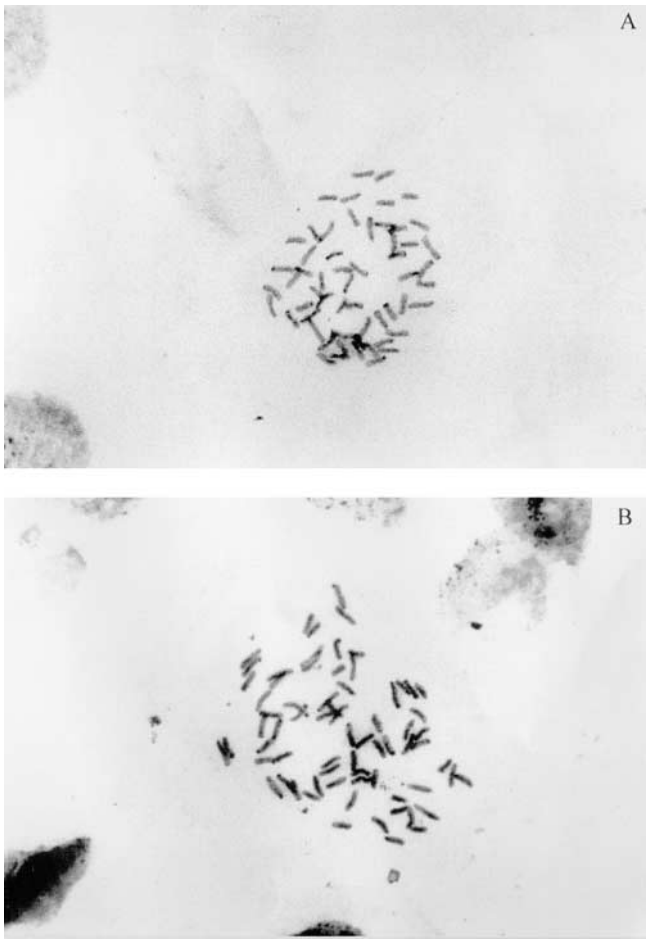

Fig. 1. *Bromus biebersteinii* chromosomes from root-tip preparations. (A) Octaploid (2*n* = 56; PI 325226); (B) Decaploid (2*n* = 70; PI 341222)

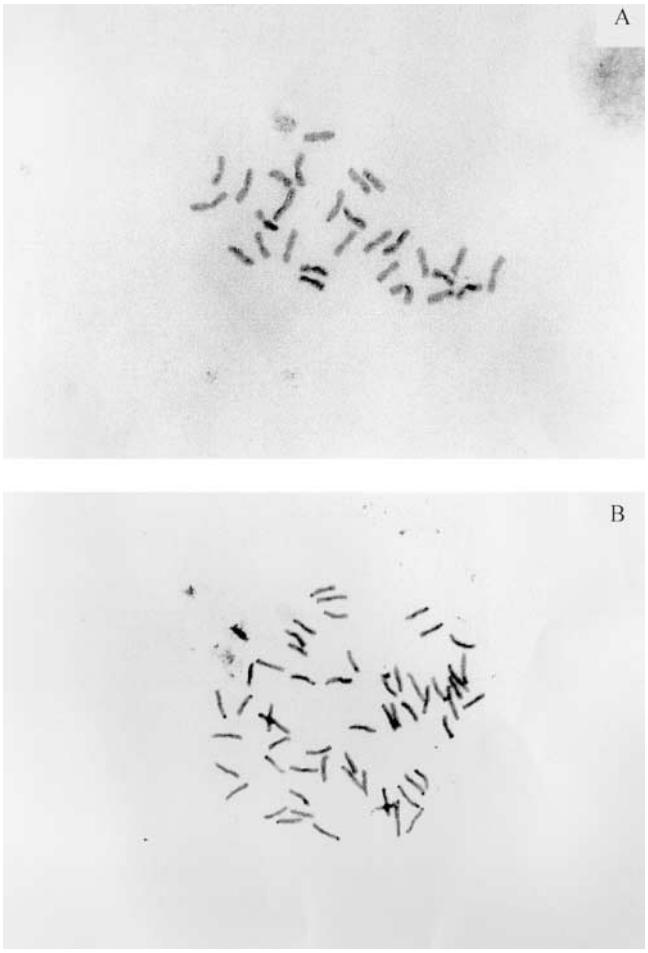

Fig. 2. *Bromus inermis* chromosomes from root-tip preparations. (A) Tetraploid (2*n* = 28; PI 315385); (B) Octaploid (2*n* = 56; Lincoln bromegrass).

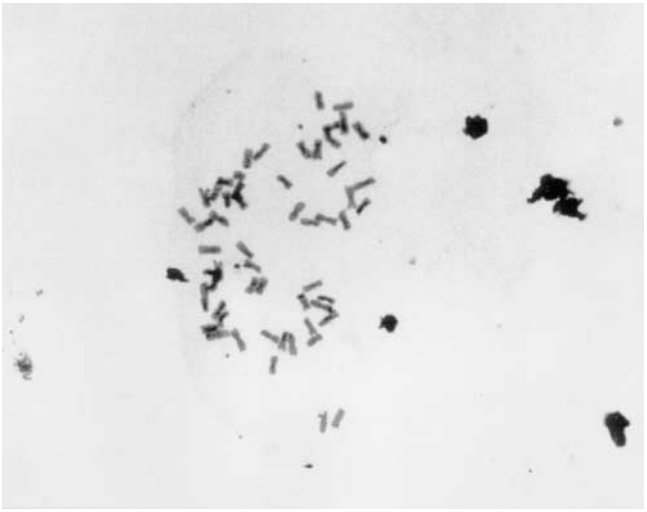

Fig. 3. Octaploid *B. inermis* ssp. *pumpellianus* chromosomes (2*n* = 56; PI 562648) from root-tip preparations.

that were studied cytologically. PI 315385 had a plant with 27 chromosomes and PI 440203 had a plant with 29 chromosomes. More potential aneuploid plants as determined by DNA content (see below) were detected in species with higher ploidy levels but accurate chromosome number counts were not attempted. Sigurbjornsson et al. (1958) and Schertz and Murphy (1958) have demonstrated that aneuploids frequently occur in the octaploid *B. inermis* L.

Bromegrass accessions were grouped into four ploidy levels based on their DNA content (Table 1). The average DNA content (2C) for diploids (2*n* = 14) and tetraploids (2*n* = 28) were 6.14 and 11.74 pg, respectively, while the octaploids (2*n* = 56) and the decaploids (2*n* = 70) had 22.31 and 26.53 pg $2C^{-1}$, respectively. Accessions for which the standard deviation (SD) for the 10 analyzed plants was greater than 1 pg, had plants that differed in ploidy as determined by DNA content or had plants with DNA contents which were intermediate to the mean DNA contents for specific ploidy levels of that species. The plants with DNA contents intermediate between two ploidy levels were classified as potential aneuploids. Therefore, accessions with SD values greater than 1 were treated as mixtures; i.e., they contained plants differing in ploidy or contained some aneuploid plants.

Most *B. inermis* (94.3%) and *B. inermis* ssp. *pumpellianus* (77.7%) accessions had a DNA content that indicated they were octaploids while the majority of the *B. riparius* (93.1%) and *B. biebersteinii* (87.5%) had a DNA content that indicated they were decaploids (Tables 1 and 2). Hexaploid chromosome numbers (2*n* = 42) have been reported for *B. inermis* (Stahlin, 1929; Knobloch, 1943; Darlington and Janaki-Ammai, 1945). However, in this study, the DNA content measurement of more than 255 *B. inermis* accessions, no plants with theoretical hexaploid DNA content were identified. This indicates that, at least in the current germplasm collection, hexaploids occur at a low frequency, if at all.

The average DNA content of the octaploid species were 22.15 pg for *B. riparius*, 22.62 pg for *B. biebersteini*, 22.72 pg for *B. inermis* ssp. *pumpellianus*, and 22.28 for *B. inermis*. The average DNA content of the four octaploid bromegrass species supports previous research which suggests that these octaploid species have a similar genomic structure and have very close genomic relationships (Pilay and Hilu, 1990; Vogel et al., 1996). Average DNA content of the decaploids was 26.07 pg for *B. biebersteini*, 26.50 pg for *B. inermis* ssp. *pumpellianus*, and 26.64 pg for *B. riparius*. These results agree

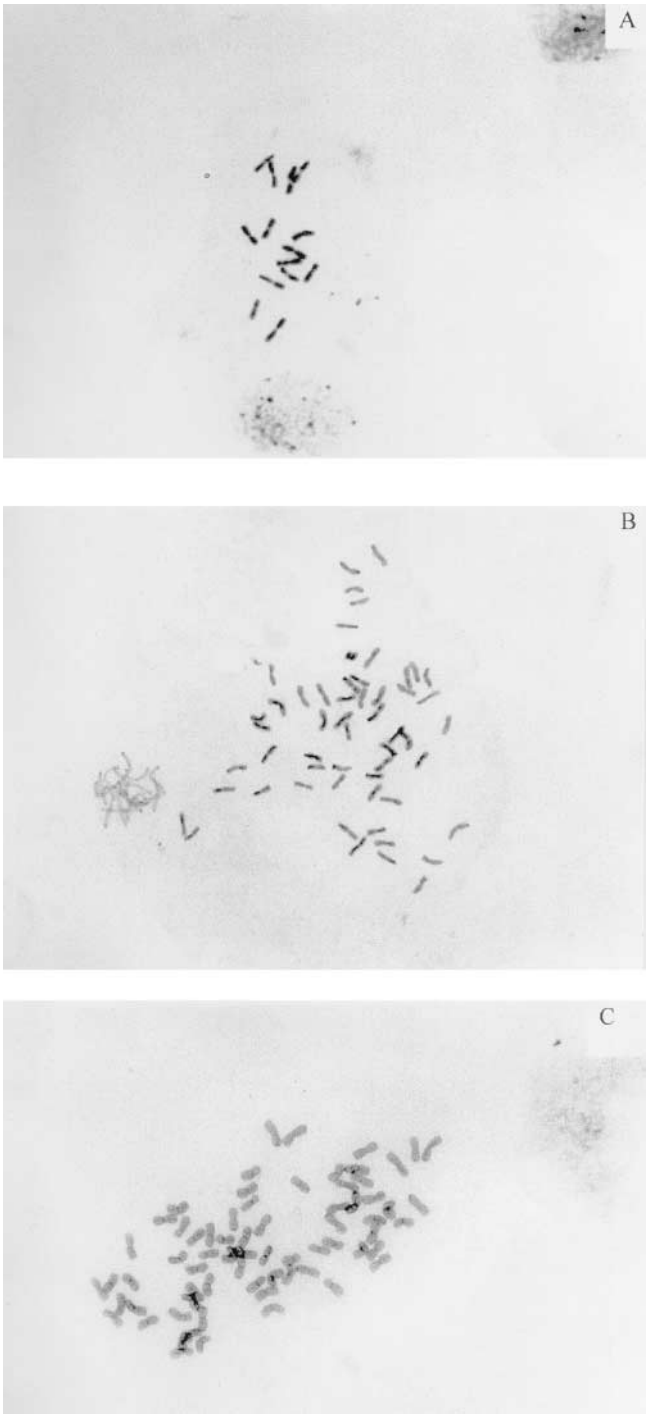

Fig. 4. *Bromus riparius* chromosomes from root-tip preparations. (A) Diploids (2*n* = 14; PI 440215); (B) Octaploid (2*n* = 56; PI 315380); (C) Decaploid (2*n* = 70; PI 536013).

Table 2. Country of origin of bromegrass accessions, number of accessions in each species, ploidy classes, and total number of accessions contributed by each country.

Country	Ploidy level					Total
	2*x*	4*x*	8*x*	10*x*	Mix†	
	n					
Bromus biebersteini						
Unknown				3		3
Former U.S.S.R.			1		1	2
Canada				3		3
Turkey				1		1
Bromus inermis* spp. *pumpellianus						
Germany			1			1
United States			4	2		6
Canada			2			2
Bromus riparius						
Former U.S.S.R.	1		2	27	5	35
Russian Federation				12		12
Canada				2		2
Bromus inermis* ssp. *inermis						
Unknown			8			8
United States			26		1	27
Turkey			21			21
Sweden			2			2
Spain			1			1
Yugoslavia			2			2
Former USSR		2	55		2	59
Poland			5		1	6
Canada			11		1	12
Germany			1			1
Romania			5			5
Australia			3			3
Norway			1			1
Bulgaria			1			1
Hungary			2			2
Iran			2			2
Japan			14		1	15
Russian Federation		1	57		1	59
Kazakhstan		3	8		1	12
China		8	5			13
Ukraine			3			3
Total	1	14	243	50	14	322

† Accessions containing aneuploid plants or mixed ploidy level.

with previous reports which suggest that decaploid bromegrasses share some genomes with octaploid bromegrasses plus have an additional genome (Schults-Schaffer, 1960).

Bennett and Smith (1976) reported the DNA content of *B. inermis* as 23.6 pg $2C^{-1}$ using Feulgen microdensitometry with *Secale cereale* L. cv. Petkus Spring as the standard. The difference between their value and the value in this report is probably due to procedural differences.

The only designated genomes in the genus *Bromus* are A and B (Armstrong, 1991). On the basis of previous cytogenetic studies tetraploid *B. inermis* has the genomic composition of AABB while the octaploid form has AAAABBBB (Hill and Carnahan, 1957; Armstrong, 1973, 1979, 1982, 1991). The A and B genomes are believed to be closely related (Armstrong, 1979). The mean DNA content of the tetraploid and octaploid forms of *B. inermis* accessions support these cytogenetic findings because octaploids had almost twice the DNA content of the tetraploids. DNA content of the tetraploid, octaploid and decaploid accessions is approximately 2, 4 and 5 times larger, respectively, than the DNA content of plants of the diploid ($2n = 14$) *B. riparius* accession, PI 440215.

The expected DNA content in octaploids was twice that of tetraploids since the copy number of the same genomes in octaploids is twice that of tetraploids. However, the average DNA content of the octaploids was 1.2 pg less than the expected amount (Table 1). These results indicate a slight tendency toward diminution of DNA content with increased ploidy. Compaction of DNA in polyploid nuclei can produce an underestimate of the DNA measurements (Verma and Rees, 1974; Kenton, 1984b) but it has also been observed in several cases where polyploids have smaller chromosomes and lower DNA content than expected (Yamaguchi and Tsunoda, 1969; Martinez and Ginzo, 1985; Poggio and Hunziker, 1986). Vogel et al. (1999) 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. The variation in DNA content among bromegrass accessions within ploidy levels and the lower than expected DNA content of the higher ploidy plants is probably due to the gain or loss of DNA content during the evolution of these species and cytotypes.

Only one diploid accession (PI 440215), a *B. riparius* accession, was found among all the accessions surveyed. It was collected in Kazakhstan (Chimkent). Additional diploid accessions of this and related species would be extremely useful in determining the origin and genomic composition of the polyploid bromegrasses and also for genetic studies which are simpler to conduct at the diploid level than the polyploid level. Only 14 tetraploid accessions were found among accessions of *B. inermis*, two of which were collected in the former USSR. The remainder of the tetraploid accessions were mainly from China and Kazakhstan. Smooth bromegrass and meadow bromegrass are of Eurasian origin and the geographic regions from which diploid and tetraploid bromegrasses were collected is in the eastern range of the bromegrasses. Thus, additional collections of diploid and tetraploid species of *B. inermis* and related species are needed from other areas of Eurasia which are currently poorly represented in the collection.

Bromus inermis accessions in USDA NPGS were collected mainly from the former USSR and the USA. Accessions from the USA and Canada are cultivars and germplasms developed from introductions. Therefore, the USDA bromegrass collection does not represent all of the geographic regions where the smooth and meadow bromegrass occurs naturally. Additional collections are needed from Europe, the Middle East, and China to increase the diversity of bromegrasses in the USDA collection.

The percentage of accessions of the other three related species (*B. biebersteinii*, *B. inermis* ssp. *pumpellianus*, and *B. riparius*) within the bromegrass collection is very low and represent only a few geographic regions. Therefore, USDA bromegrass germplasm could be enhanced by collections from western and central Europe, the Middle East, and China to provide breeders with a larger diversity of germplasm for use in breeding programs.

The chromosome numbers of the accessions evalu-

ated in this are now available on the USDA's National Plant Germplasm Systems GRIN database (http://www.ars-grin.gov/npgs/).

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