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2005

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Metin Tuna
University of Trakya, mtuna@tu.tzf.edu.tr

Kenneth P. Vogel
University of Nebraska-Lincoln, kvogel1@unl.edu

K. Arumuganathan
Benaroya Research Institute

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Euphytica (2005) 146: 177–182
DOI: 10.1007/s10681-005-5341-6
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Genome size and Giemsa C-banded karyotype of tetraploid *Bromus ciliatus* L.

Metin Tuna[1,*], Kenneth P. Vogel[2] & K. Arumuganathan[3,4]
[1]*Department of Agronomy, Tekirdag Agriculture Faculty, University of Trakya, Tekirdag, Turkey;* [2]*USDA-ARS, Wheat, Sorghum, and Forage Research Unit, 344 Keim Hall, University of Nebraska, P.O. Box 830937, Lincoln, NE 68507-0937;* [3]*Center for Biotechnology, University of Nebraska, Lincoln, NE 68588;* [4]*Present address: Virginia Mason Research Center, Benaroya Research Institute, 1201 Ninth Avenue, Seattle, WA 98101, USA*
*(*author for correspondence: e-mail: mtuna@tu.tzf.edu.tr)*

Received 29 December 2004; accepted 12 April 2005

Key words: Bromus, genome size, karyotypes, nuclear DNA content, C-banding, taxonomy

Summary

Tetraploid *Bromus ciliatus* L. is a North American bromegrass that has been placed in the *Pnigma* section of *Bromus*. The objective of this study was to characterize the genome of tetraploid *B. ciliatus* by cytogenetic methods and compare it to the genomes of other species included in the section *Pnigma*. All the plants of the accession (USDA PI 232214) selected for chromosome counting were tetraploids ($2n = 28$). The mean 2C nuclear DNA content for tetraploid *B. ciliatus* was 19.13 ± 0.07 pg as determined by flow cytometry which is significantly greater than the tetraploid DNA content of *B. inermis* Leyss. (11.74 ± 0.16 pg). C-banding procedures were used to identify individual mitotic chromosomes and to develop a karyotype for *B. ciliatus*. The genome of the tetraploid *B. ciliatus* consisted of 16 median chromosomes, eight submedian chromosomes, and four chromosomes with satellites which included one pair with a large satellite and one pair with a small satellite. The general pattern of the distribution of constitutive heterochromatin in *B. ciliatus* was quite different than the other bromegrasses that have been analyzed to date. Except for two pairs of chromosomes, all chromosomes in tetraploid *B. ciliatus* had telomeric bands on one or both arms. Some of the chromosomes with telomeric bands had centromeric bands that were located at one or both sides of the centromere and intercalary bands which were generally absent in the other bromegrass species. It was possible to identify all chromosomes of tetraploid *B. ciliatus* and to match the pairs of homologous chromosomes by using chromosome lengths, arm length ratios and C-banding patterns. The results of this study indicate that tetraploid *B. ciliatus* has different genomes than the European species evaluated to date in the section *Pnigma*.

Abbreviations: Chr, chromosome; NOR, nucleolus organizer region; pg $2C^{-1}$, DNA content of a diploid somatic nucleus in picogram; Sat, satellite; SD, standard deviation

Introduction

The species of the genus *Bromus* L. are cool-season grasses. The genus *Bromus* contains more than 100 species distributed over all continents (Gould & Shaw, 1983). There has been no consensus on the systematic treatment of species of the *Bromus* complex. The genus has been divided variously into six sections (Smith, 1970), seven subgenera (Stebbins, 1981), or five genera (Tsvelev, 1976). Tetraploid *Bromus ciliatus* L. is a North American *Bromus* species that has been placed in the *Pnigma* section of *Bromus*. The section contains approximatelly 60 species (Smith, 1970) that are found throughout Eurasia and America. A comparatively wide range in chromosome size exists among the species of *Bromus* section *Pnigma*. All indigenous

American species have a larger chromosome size than those from Eurasia (Armstrong, 1983). Armstrong (1983) suggested that North American species of section *Pnigma* might be a distinct group of species that could be separated from the other species of the section on the basis of chromosome size. Genomic relationships between and within sections of genus *Bromus* have not been investigated in detail. Earlier systematic investigations within the genus are based on morphology, crossability and analysis of chromosome pairing in artificially produced inter- and intra-generic hybrids.

Because many species in a genus have the same chromosome numbers, differences in nuclear DNA content can be used to delimit intrageneric divisions (Ohri, 1998). Giemsa C-banding technique, which stains constitutive heterochromatin, is a technique that has been used successfully in many species to identify individual chromosomes and establish genomic relationships among species (Fominaya et al., 1988; Gill & Sears, 1988; Tayyar et al., 1994; Falistocco et al., 1995; Tuna et al., 2004).

The objectives of this study were to characterize the genome of tetraploid *Bromus ciliatus* L. for the first time for nuclear DNA content, develop a karyotype using C-banding analysis, and utilize this information to compare its genome to the genomes of Eurasian species included in the section *Pnigma* of the genus *Bromus*.

Material and methods

The seeds of tetraploid *B. ciliatus* accession, PI 232214, were obtained from the United States Department of Agriculture's (USDA) National Plant Germplasm System (http://www.ars-grin.gov/npgs/) via the USDA Regional Plant Introduction Station, Pullman, WA. The seeds were germinated in germination boxes containing germination paper saturated with distilled water. For flow cytometric analysis twenty seedlings were transferred to pots filled with a mixture of soil, perlite and peat moss (2:1:1, v/v/v). The plants were grown in a greenhouse and exposed to a 16-h photoperiod. Nuclear DNA content of 10 individual plants was determined using flow cytometry at the University of Nebraska Flow Cytometry Core Research Facility [FACScan flow cytometer, Becton Dickinson Immunocytometry system, San Jose, CA]. Hexaploid wheat, *Triticum aestivum* cv. Araphoe, which has a 2C complement of 34.68 pg of DNA per nucleus, was used as the standard. The procedures that were used are described in detail in Tuna et al. (2001b).

For cytological investigations, imbibed seeds in a germination box were kept at room temperature for 1 day before they were transferred to a refrigerator at 0 to 4 °C for one to a few days (until the majority of seeds appeared to be germinating). Seeds were then placed in the dark at room temperature and fast growing root tips were collected when they reached 1–1.5 cm in length. Harvested root tips were treated with 0.05% colchicine (w/v) for 1–2 h and stored in a fixative of ethanol:glacial acetic acid (3:1, v/v) for at least two weeks before making slide preparations. Techniques for the chromosome squash preparations and C-banding are described in detail in Tuna et al. (2001a). Cells with well-spread chromosomes were identified and an image of each cell was captured by a Spot I digital camera (Diagnostic Instruments Inc.). Printed enlarged pictures (3000×) of ten cells from 10 different plants with well-spread metaphase chromosomes were used for analysis and construction of karyotypes.

Chromosome measurements were made on the enlarged prints by ruler and converted to microns by relating measurements from enlarged prints with measurements made in a microscope with a micrometer. The chromosomes were identified on the basis of their total length, arm-ratio, C-banding patterns, and presence or absence of satellites.

Results

The mean 2C nuclear DNA content for *B. ciliatus* was 19.13 ± 0.07 pg. All the plants of the accession selected for chromosome counting were tetraploids with $2n = 28$ mitotic chromosomes. No aneuploids were found.

Mitotic metaphase chromosomes of tetraploid *B. ciliatus* before and after the C-banding procedure are shown in Figures 1 and 2, respectively. A detailed C-banded karyotype based on this specific cell is presented in Figure 3 and accompanying information on total chromosome lengths and arm length ratios are listed in Table 1. The chromosomes ranged in length from 9.44 to 13.07 μm. The total haploid genome length was 158.90 μm. Arm length ratio of tetraploid *B. ciliatus* ranged between 1.01 and 1.98. The genome of tetraploid *B. ciliatus* consisted of 16 median chromosomes, eight submedian chromosomes and four chromosomes with satellites, including one pair with a large satellite and one pair with a small satellite.

The general pattern of the distribution of constitutive heterochromatin in tetraploid *B. ciliatus* was quite

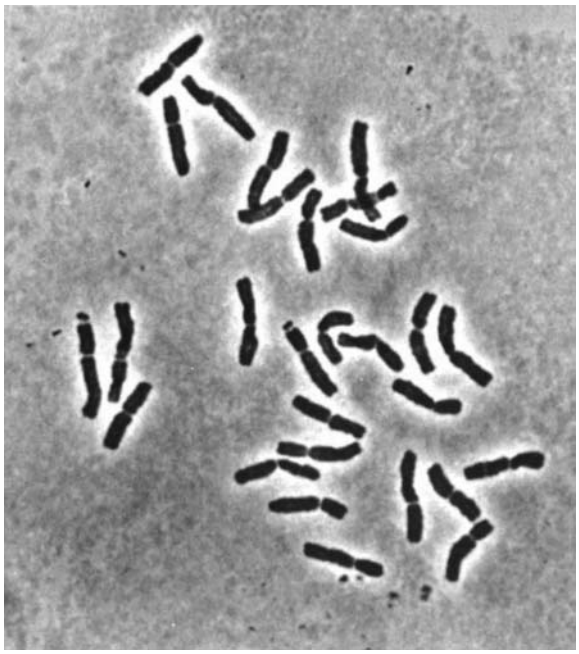

Figure 1. Mitotic chromosomes of *B. ciliatus* L. (before C-banding).

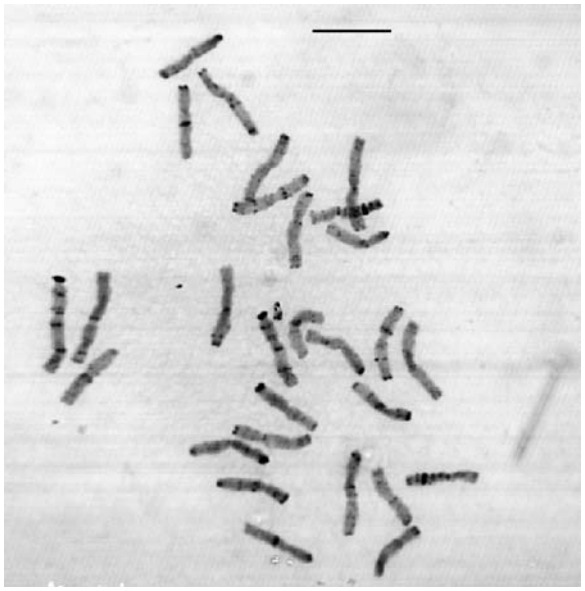

Figure 2. C-banded mitotic chromosomes of *B. ciliatus* L. (bar is 10 μm in length).

different than the other bromegrasses that have been analyzed to date using C-banding techniques. Except for two pairs of chromosomes, all chromosomes in tetraploid *B. ciliatus* had telomeric bands on one or both arms. Some of the chromosomes with telomeric

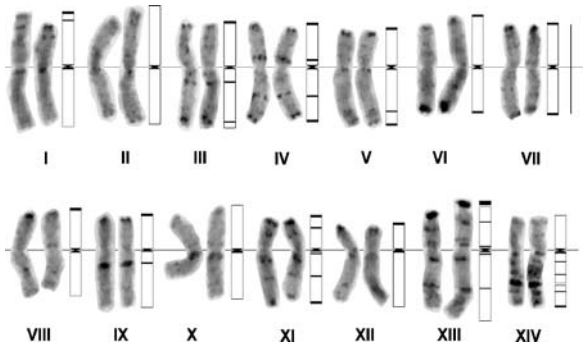

Figure 3. C-banded karyotype of tetraploid *B. ciliatus* L. (bar is 10 μm in length).

bands also had centromeric bands that were located at one or both sides of the centromere and intercalary bands which are generally absent in other bromegrass species. Based on chromosome lengths, arm length ratios and C-banding patterns, it was possible to identify all chromosomes of tetraploid *B. ciliatus* and to match the pairs of homologous chromosomes. The banding patterns and morphology of the 14 somatic chromosome pairs are described below.

Chromosome group I was the longest with a telomeric band on the short arm. The chromosomes had one centromeric band on the long arm and a faint distal C-band on the short arm. Chromosomes of the group had a median centromere with an arm length ratio of 1.18. Centromeric C-bands together with faint distal C-bands enabled these chromosomes to be distinguished from others in the complement.

Chromosome group II had a total length of 12.90 μm, a median centromere, and an arm length ratio of 1.02. The chromosomes of this group were the only ones with no C-bands. This feature enabled these chromosomes to be distinguished from others in the complement.

Chromosome group III had a total length of 12.48 μm, a median centromere, and an arm length ratio of 1.15. The chromosomes of this group had one distal and one proximal C-band on the long arm in addition to a faint telomeric C-band on the short arm. Location of distal and proximal C-bands on the long arm enabled these chromosomes to be distinguished from others in the complement.

Chromosome group IV had a total length of 11.83 μm, a median centromere, and an arm length ratio of 1.28. The chromosomes of this group had one distal C-band on the long arm and one proximal C-band

Table 1. Chromosomes of the tetraploid *Bromus ciliatus*

Chr.	Long arm Mean ± S.D. (μm)	Short arm Mean ± S.D. (μm)	Total length Mean ± S.D. (μm)	Sat. size Mean ± S.D. (μm)	Arm ratio[1] (Mean ± S.D.)	Chr. Type
1	7.09 ± 1.01	5.98 ± 0.63	13.07 ± 1.57		1.18 ± 0.13	Median
2	6.56 ± 0.50	6.33 ± 0.35	12.90 ± 0.86		1.02 ± 0.02	Median
3	6.66 ± 0.50	5.81 ± 0.49	12.48 ± 0.74		1.15 ± 0.12	Median
4	6.66 ± 0.49	5.17 ± 0.14	11.83 ± 0.48		1.28 ± 0.11	Median
5	7.36 ± 0.62	4.29 ± 0.50	11.65 ± 1.10		1.72 ± 0.09	Submedian
6	5.76 ± 0.39	5.38 ± 0.47	11.14 ± 0.85		1.06 ± 0.03	Median
7	6.22 ± 0.45	4.92 ± 0.23	11.14 ± 0.70		1.26 ± 0.03	Median
8	5.49 ± 0.20	5.22 ± 0.44	10.72 ± 0.63		1.05 ± 0.05	Median
9	6.87 ± 0.19	3.46 ± 0.08	10.34 ± 0.10		1.98 ± 0.10	Submedian
10	5.19 ± 0.72	5.15 ± 0.36	10.34 ± 0.77		1.01 ± 0.01	Median
11	6.62 ± 0.33	3.63 ± 0.32	10.25 ± 0.66		1.82 ± 0.06	Submedian
12	6.23 ± 0.46	3.20 ± 0.40	9.44 ± 0.86		1.95 ± 0.11	Submedian
13	7.91 ± 0.56	4.09 ± 0.58	13.22 ± 1.18	1.21 ± 0.11	1.49 ± 0.05	Satellite
14	4.30 ± 0.31	3.39 ± 0.35	10.43 ± 0.63	2.74 ± 0.23	1.42 ± 0.18	Satellite
Total genome length			317.80			

S.D. = Standard deviation.
Median = Arm ratio is lower than 1.50.
[1] Arm ratio = Length of the long arm divided by length of the short arm.

on the short arm in addition to telomeric C-bands on both arms. The C-banding pattern of the chromosomes enabled them to be distinguished from others in the complement.

Chromosome group V had a total length of 11.65 μm, a submedian centromere, and an arm length ratio of 1.72. The chromosomes of this group had one faint centromeric C-band, one faint distal C-band and one faint telomeric C-band on the long arm, and one large telomeric C-band on the short arm. These features enabled these chromosomes to be distinguished from others in the complement.

Chromosome group VI had a total length of 11.14 μm, a median centromere, and an arm length ratio of 1.06. The chromosomes of this group had one faint telomeric C-band on one of the arms, a large telomeric C-band on the other arm, and no intercalary bands. The large telomeric C-bands of these chromosomes were the largest C-bands in the karyotype of tetraploid *B. ciliatus*. These features enabled these chromosomes to be distinguished from others in the complement.

Chromosome group VII had a total length of 11.14 μm, a median centromere, and an arm length ratio of 1.26. The chromosomes of this group had telomeric C-bands on both arms and they did not have any intercalary bands.

Chromosome group VIII had a total length of 10.72 μm, a median centromere, and an arm length ratio of 1.05. The chromosomes of this group had a telomeric C-band on the short arm and they did not have any intercalary bands.

Chromosome group IX had a total length of 10.34 μm, a submedian centromere, and an arm length ratio of 1.98. The chromosomes of this group had telomeric C-bands on the short arm and a large proximal C-band on the long arm. The large proximal C-band enabled these chromosomes to be distinguished from others in the complement.

Chromosome group X had a total length of 10.34 μm, a median centromere, and an arm length ratio of 1.01. The chromosomes of this group were the second chromosome group of the karyotype that did not have a telomeric C-band but they did have a centromeric C-band. These features enabled these chromosomes to be distinguished from others in the complement.

Chromosome group XI had a total length of 10.25 μm, a submedian centromere, and an arm length ratio of 1.82. The chromosomes of this group had one distal C-band on the short arm, one proximal C-band and one faint distal C-band on the long arm, and telomeric C-bands on both arms. Distribution of intercalary bands enabled chromosomes of this group to be distinguished from others in the complement.

Chromosome group XII had a total length of 9.44 μm, a submedian centromere, and an arm length ratio of 1.95. The chromosomes of this group had one faint centromeric C-band and a telomeric C-band on the short arm.

Chromosome group XIII consists of two chromosomes with small satellites. Chromosomes of this group had a total length of 13.22 μm and an arm length ratio of 1.49. The chromosomes of the group had one distal and one proximal C-band on both arms and a telomeric C-band on the short arm, which also carried the satellite. These features enabled these chromosomes to be easily distinguished from others in the complement.

Chromosome group XIV consists of two chromosomes with large satellites. Chromosomes of this group had a total length of 10.43 μm and an arm length ratio of 1.42. The chromosomes had one centromeric C-band, one telomeric C-band and three intercalary C-bands on the long arm, which also carried the large satelite. These features enabled these chromosomes to be distinguished easily from others in the complement.

Discussion

Nuclear DNA contents of various species of genus *Bromus* have been reported (Benneth & Smith, 1976; Naganowska, 1993; Tuna et al., 2001b; Joachimiak et al., 2001). However, the nuclear DNA content of *Bromus ciliatus* was unknown prior to this study. The 2C nuclear DNA content of tetraploid *B. ciliatus* L. ($2n = 28$) was 19.13 ± 0.07 pg, which differs significantly from the tetraploid DNA content of Eurasian species of *Bromus* section *Pnigma*. Tuna et al. (2001b) reported that diploid, tetraploid, octoploid and decaploid plants of various Eurasian species of *Bromus* section *Pnigma* had average 2C nuclear DNA content values of 6.14, 11.74, 22.31 and 26.53 pg, respectively. They reported that Eurasian *Bromus* species with the same ploidy levels in the section *Pnigma* had very similar nuclear DNA contents. For example, octoploid *B. riparis*, *B. biebersteini*, *B. inermis* and *B. inermis ssp. pumpellianus* had 2C nuclear DNA contents of 22.15, 22.28, 22.62 and 22.72 pg, respectively. They also reported a linear correlation between nuclear DNA content and ploidy level in the section. The nuclear DNA contents of the tetraploid, octoploid and decaploid accessions were approximately 2, 4, and 5 times larger, respectively, than the DNA content of diploid accessions (Tuna et al., 2001b). These results indicate that Eurasian *Bromus* species in section *Pnigma* have similar genomes, which is expected because they are close relatives and are included in the same section taxonomically. However, 2C nuclear DNA content of *B. ciliatus* (19.13 pg) is much larger than that of tetraploid *Bromus inermis* (11.74 pg) which has the same chromosome number. The nuclear DNA content difference between tetraploid *B. ciliatus* and tetraploid *B. inermis* is about 7.5 pg, which is over 7300 Mbp when expressed on a DNA base pair basis. The large difference in DNA content between *B. ciliatus* and Eurasian species of section *Pnigma* indicates that they have different genomes.

All the investigated plants of *B. ciliatus* had tetraploid chromosome numbers ($2n = 28$). These results are in agreement with previous reports (Elliott, 1949; Wilton, 1965; Armstrong, 1981, 1983; Pillay & Hilu, 1990; Elliott, 1949; Barnett, 1955). All the plants used in the study had stable chromosome numbers in root meristem cells. Such somatic stability has been observed in *Bromus* species with low ploidy levels (Joachimiak et al., 2001; Tuna et al., 2001b) while occasional aneuploid plants were found in *Bromus* species with higher ploidy levels (Sigurbjörnsson et al., 1958; Schertz & Murphy, 1958; Joachimiak et al., 2001; Tuna et al., 2001b).

Chromosomes of the species of *Bromus* are generally median to submedian with regard to position of the centromere (Armstrong, 1991). In this regard, chromosome morphology of the *B. ciliatus* plants evaluated in this study is in agreement with previous reports. However, the mean length of *B. ciliatus* chromosomes (approximatelly 11.30 μm) was almost double that of Eurasian species (approximately 6.10 μm) of *Bromus* section *Pnigma*. Large differences in chromosome size between North American and Eurasian species of *Bromus* section *Pnigma* have been reported in previous studies as well (Barnett, 1955; Stebbins, 1981; Armstrong, 1981, 1982, 1983). Armstrong (1983) suggested that changes in DNA content have played a role in the evolution of *Bromus* genomes and that the observed differences in chromosome size reflected differences in nuclear DNA quantity.

Giemsa C-banding has not been employed in detail to characterize genomes in the genus *Bromus*. However, all *Bromus* species analyzed previously had similar C-banding patterns consisting mainly of telomeric C-bands (Armstrong, 1991; Kula, 1999; Tuna et al., 2001a,2004; Joachimiak et al., 2001). However, a number of large interstitial C-bands together with telomeric bands were observed in *B. ciliatus* and its C-banding pattern differed from other species of *Bromus* studied previously. The C-banding patterns together

with chromosome morphology allowed all homologous chromosome pairs to be identified in tetraploid *B. ciliatus*. The karyotype developed for *B. ciliatus* (Figure 3) clearly shows that it has 14 groups of chromosome pairs indicating that the species is allotetraploid.

Giemsa C-banding is a technique that stains constitutive heterochromatin. The differences in constitutive heterochromatin pattern between tetraploid *B. ciliatus* and European species of section *Pnigma* indicate differences in DNA sequences among the species. The results of nuclear DNA content and C-banding analysis support Armstrong's (1984) suggestion that the North American species of section *Pnigma* may be a distinct group of species in the section. Based on hybridization studies, genetic isolation barriers are, in general, strong between species with large (North American) and small (Eurasian) chromosomes of *Bromus* section *Pnigma* (Armstrong, 1983). Further work on more species will be required to show whether the section is monophyletic or an artificial assemblage of species.

In conclusion, the genome size of *B. ciliatus*, a North American tetraploid, was determined and a complete karyoptype was developed using C-banding and chromosome morphology, which clearly indicates that the species is an allotetraploid. Based on the results of this study, tetraploid *B. ciliatus* L. has different genomes than the European species of the *Bromus* section *Pnigma*.

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