

Karyotype characterization and comparison of three hexaploid species of *Bromus* Linnaeus, 1753 (Poaceae)

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Abstract

Chromosome morphometry and nuclear DNA content are useful data for cytotaxonomy and to understand the evolutionary history of different taxa. For the genus *Bromus* Linnaeus, 1753, distinct ploidy levels have been reported, occurring from diploid to duodecaploid species. The geographic distribution of *Bromus* species has been correlated with chromosome number and ploidy level. In this study, the aims were to determine the nuclear genome size and characterize the karyotype of the South American *Bromus* species: *Bromus auleticus* Trinius ex Nees, 1829, *Bromus brachyanthera* Döll, 1878 and *Bromus catharticus* Vahl, 1791. The mean nuclear 2C value ranged from 2C = 12.64 pg for *B. catharticus* to 2C = 17.92 pg for *B. auleticus*, meaning a maximum variation of 2C = 5.28 pg, equivalent to 41.70%. Despite this significant difference in 2C value, the three species exhibit the same chromosome number, 2n = 6x = 42, which confirms their hexaploid origin. Corroborating the genome size, the chromosome morphometry (total, short- and long-arm length) and, consequently, the class differed among the karyotypes of the species. Based on the first karyograms for these *Bromus* species, some morphologically similar and several distinct chromosome pairs were found. Therefore, the karyotype characterization confirmed the hexaploid origin of the studied *Bromus* species, which differ in relation to the karyogram and the nuclear 2C value. Considering this, cytogenetics and flow cytometry can be used to discriminate *Bromus* species, contributing to taxonomy and systematic studies and providing information on the evolutionary history of this taxa.

Keywords

Karyogram, nuclear genome size, polyploidy, forage grasses

Introduction

The genus *Bromus* Linnaeus, 1753, family Poaceae comprises more than 160 species of annual and perennial grasses (Acedo and Lamas 2001). This taxon is widely distributed around the world (Williams et al. 2011), demonstrating the adaptability of its species (Martinello and Schifino-Wittmann 2003). The genus *Bromus* includes important forage grasses, such as *Bromus auleticus* Trinius ex Nees, 1829, *Bromus brachyanthera* Döll, 1878 and *Bromus catharticus* Vahl, 1791 (Puecher et al. 2001, Martinello and Schifino-Wittmann 2003, Iannone et al. 2010).

The basic chromosome number of the genus *Bromus* is $x = 7$, and its species possess karyotypes varying from $2n = 2x = 14$ (diploid) to $2n = 12x = 84$ (duodecaploid) (Fedorov 1969, Armstrong 1984, 1987, Klos et al. 2009, Williams et al. 2011). Most of the species are diploid ($2n = 2x = 14$) or tetraploid ($2n = 4x = 28$) (Martinello and Schifino-Wittmann 2003), but large variation in chromosome number among *Bromus* species has been found, such as: *Bromus cappadocicus* Boissier et Balansa, 1857, and *B. tomentosus* Trinius, 1813, with $2n = 2x = 14$; *B. erectus* Huds., 1762, *B. biebersteinii* Roemer et Schultes, 1817, and *B. stenostachyus* Boissier, 1884, with $2n = 4x = 28$; *B. tomentellus* Boissier, 1846, *B. variegatus* M. Bieberstein, 1819 (Sheidai et al. 2008), *B. auleticus* (Martinello and Schifino-Wittmann 2003), *B. bonariensis* Parodi et J. H. Camara, 1963, *B. brevis* Steudel, 1854, *B. parodii* Covas et Itria, 1968, *B. brachyanthera* and *B. catharticus* Vahl, 1791 (Schifino and Winge 1983, Naranjo 1985) with $2n = 6x = 42$. Variation in chromosome number has also been found within the same species, such as in *Bromus kopetdagensis* Drobow, 1925, ($2n = 6x = 42$ in Tehran and $2n = 10x = 70$ in Emamzadeh-Hashem; Sheidai et al. 2008) and in *Bromus setifolius* J. Presl, 1830, ($2n = 10x = 70$ for ‘Pictus’ and ‘Brevifolius’, and $2n = 4x = 28$ for ‘Setifolius’; García et al. 2009). So, cytogenetic and plant morphology data supported the classification of the *B. setifolius* lines as separate species (García et al. 2009).

Karyotype characterization showed that the chromosomes of the *Bromus* species are similar in relation to total length and class (Joachimiak et al. 2001), with the occurrence of metacentric and submetacentric chromosomes being reported (Sheidai et al. 2008). This way, the distinction between karyotypes of *Bromus* species with the same chromosome number is generally carried out based on the size of the satellite portions (Armstrong 1983, Joachimiak et al. 2001) and heterochromatin distribution (Klos et al. 2009).

Stebbins (1981) reported that the genus *Bromus* originated in Eurasia. During the Pliocene, three sections were originated: *Ceratochloa* P. Beauvois, 1812, *Pnigma* Dumort, 1823 and *Neobromus* Shear, 1900, being that *Ceratochloa* and *Neobromus* spread to

Americas. Given this hypothesis, Stebbins argued that the geographic region Eurasia was also the differentiation center of diploid, tetraploid and, most likely, hexaploid species.

Differentiation of allohexaploid *Bromus* species in South America proceeded in the Pleistocene. Meanwhile, in North America, allopolyploidy events also occurred, leading to new ones with higher ploidy level (8x, 12x) (Stebbins 1947, 1981). This way, the *Bromus* species in South America is somewhat restricted to hexaploid species, differently from those found in North America, which are octoploids and duodecaploids (Massa et al. 2004).

According to current knowledge, the South American species (as *B. auleticus*, *B. brachyanthera* and *B. catharticus*) have chromosome number of $2n = 6x = 42$ (Schifino and Winge 1983, Naranjo 1985, Martinello and Schifino-Wittmann 2003). Nevertheless, it is still necessary to confirm and understand the chromosomal changes among these species. Because chromosomal changes constitute an important mechanism of diversification and speciation (Stace 2000, Peer et al. 2009, Weiss-Schneeweiss et al. 2013), the investigation of this aspect in *Bromus* species of South America may generate knowledge on the speciation processes in this genus.

Numerical and structural chromosomal rearrangements have been reported to trigger changes in karyotype in various plant taxa. Due to these changes, the nuclear genome size varies between phylogenetically related species (Raskina et al. 2008, Bonifácio et al. 2012). Thus, nuclear DNA content measurements have increasingly been employed in taxonomic, systematic and evolutive approaches using flow cytometry (FCM). In addition to its practicality and reproducibility, FCM is useful to reveal differences among taxa, especially those that exhibit conserved chromosome number (Mabuchi et al. 2005). The nuclear genome size was measured for *Bromus* hexaploid ($2n = 6x = 42$) species. In spite of the same chromosome number, the seven species showed distinct nuclear 2C values, varying from $2C = 12.72$ pg in *Bromus willdenowii* Kunth, 1829, to $2C = 15.10$ pg in *Bromus lithobius* Trin., 1836 (Klos et al. 2009).

Hence, karyotype and nuclear 2C value are relevant data for the taxonomy and systematics of *Bromus*, as well as for understanding the evolutionary history of the genus and the relationships within the taxa. Thus, the aims of the present study were to measure the nuclear 2C value, determine the chromosome number and characterize the karyotype of the South American *Bromus* species *B. auleticus*, *B. brachyanthera* and *B. catharticus*.

Material and methods

Plant samples

Seeds of *B. auleticus*, *B. brachyanthera* and *B. catharticus* were provided by the South Forage Germplasm Bank (BAG) of Embrapa South Livestock, Brazil (BRA 00059183-4, 00080317-1 and 00059197-4, respectively). The seed samples were collected from several individuals of each species, occurring in the Brazilian Pampa biome, state of Rio Grande do Sul, Brazil. Copies of the species were deposited in the Herbarium CNPO

Embrapa (voucher numbers CNPO 4408 for *B. auleticus*, CNPO 4412 for *B. brachyanthera*, and CNPO 4408 for *B. catharticus*).

Nuclear 2C DNA measurement

Nuclear suspensions were prepared from leaf fragments (2 cm²) obtained from each specimen of *B. auleticus*, *B. brachyanthera* or *B. catharticus* (samples), together with the internal standard *Pisum sativum* L. (2C = 9.16 pg; Praça-Fontes et al. 2011). For nuclear extraction, chopping was performed in 0.5 mL of OTTO-I buffer (Otto 1990) supplemented with 2 mM dithiothreitol and 50 µg mL⁻¹ RNase. Next, 0.5 mL of OTTO-I buffer was added, and the suspensions were filtered through nylon mesh of 30 µm, placed in microtube and centrifuged at 100 *xg* for 5 min. The pellet was resuspended in 100 µl OTTO-I buffer and incubated for 10 min (Praça-Fontes et al. 2011). The nuclei suspensions were stained with 1.5 ml of OTTO I:OTTO II (1:2 – Otto 1990; Praça-Fontes et al. 2011) supplemented with 2 mM dithiothreitol, 50 µg ml⁻¹ propidium iodide and 50 µg ml⁻¹ RNase (Praça-Fontes et al. 2011). The suspensions were kept in the dark for 30 min, filtered through a 20-µm nylon mesh, and analyzed on a flow cytometer Partec PAS II / III (Partec GmbH, Germany) equipped with a laser source (488 nm). For determination of the nuclear DNA content, histograms were analyzed with the Max Partec Flow software tools. Six independent repetitions, accounting for more than 10,000 nuclei, were carried out in each analysis. The genome size of *Bromus* species was calculated according to the formula:

$$2C_D = \left(\frac{C_1}{C_2} \right) \cdot 2C_S$$

Wherein: 2C_D: value of 2C DNA content (pg) of each *Bromus* species; C₁: average G₀/G₁ peak channel of the *Bromus* species; C₂: average G₀/G₁ peak channel of *P. sativum*; 2C_S: value of 2C DNA content of *P. sativum* (2C = 9.16 pg).

Karyotype characterization and morphometric analysis

The seeds were aseptically scarified, disinfested, inoculated into medium composed of half-strength MS salts, 10 ml l⁻¹ MS vitamins (Murashige and Skoog 1962), 30 g l⁻¹ sucrose and 2.8 g l⁻¹ Phytigel, and grown in photoperiod of 16 h at 25 ± 2°C. Roots of the seedlings were excised and treated with 4 µM amiprofos-methyl (APM, Sigma) for 4 h (B.O.D., 30°C). Root apical meristems were washed in dH₂O, fixed in methanol:acetic acid (Merck®) solution (3:1), and stored at -20°C. After 24 h, the meristems were washed in dH₂O and macerated in enzyme solution pool (96.6% pectinase, 0.4% hemicellulose, 1.0% macerozyme and 4.0% cellulose, Sigma®) 1:20 (pool:dH₂O) for 2 h at 34°C. Again, the meristems were washed with dH₂O, fixed,

and stored at -20°C . Subsequently, slides were prepared by dissociation of the macerated meristems and air-drying (Carvalho et al. 2007). The slides were stained with 5% Giemsa (Merck®) for 6 min, washed twice in dH_2O , and dried under heating plate. All slides were examined under a microscope Nikon Eclipse Ci-S (Nikon). Mitotic images were captured with the objective 100x and CCD camera (Nikon Evolution™) coupled to the microscope. Morphometric analysis of the chromosomes of the three *Bromus* species was performed for the total length, length of the long and short arms, arm ratio, and chromosome class. The latter was determined as proposed by Levan et al. (1964) and reviewed by Guerra (1986).

Results

FCM nuclear suspensions resulted in G_0/G_1 fluorescence peaks with a coefficient of variation of less than 5% for *Bromus* species and *P. sativum*. Thereby, FCM procedures provided suspensions with adequate amount of isolated, intact and stoichiometrically stained nuclei. The 2C nuclear DNA content was measured for the *Bromus* species through analysis of the histograms. 2C value of *B. catharticus* was $2C = 12.64 \pm 0.00$ pg, *B. brachyanthera* was $2C = 16.73 \pm 0.16$ pg, and *B. auleticus* was 17.92 ± 0.44 pg. Mean value for *B. auleticus* was 41.70% higher than for *B. catharticus*, and 7.10% greater than for *B. brachyanthera*. In turn, *B. brachyanthera* presented 2C value 32.36% higher than that of *B. catharticus*. These values reflect interspecific variation among the nuclear genome sizes of the analyzed species.

Root meristems treated with amiprophos-methyl and macerated in enzyme pool solution resulted in adequate metaphase chromosomes. Metaphases were chosen based on the following criteria: well-spread chromosomes with well-defined constriction, no chromatin deformations and no cytoplasmic background noise. These features allowed accurate chromosome counting, karyotype measurements, chromosome class determination and karyogram assembly. All three *Bromus* species showed a conserved number of $2n = 42$ chromosomes (Figure 1).

Based on the morphometric data, the chromosome class was determined and the differences between the karyotypes of the three species were verified. *B. auleticus* presented eleven metacentric (1, 3, 4, 5, 6, 8, 9, 11, 15, 18 and 19) and ten submetacentric chromosomes (2, 7, 10, 12, 13, 14, 16, 17, 20 and 21). *B. brachyanthera* exhibited 13 metacentric (1, 2, 4, 5, 6, 9, 10, 13, 15, 16, 17, 19 and 21) and eight submetacentric chromosomes (3, 7, 8, 11, 12, 14, 18 and 20). Finally, *B. catharticus* displayed eleven metacentric (1, 4, 5, 7, 8, 9, 10, 11, 14, 17 and 20) and ten submetacentric chromosomes (2, 3, 6, 12, 13, 15, 16, 18, 19 and 21) (Table 1). Groups of morphologically similar chromosome pairs were also identified for all *Bromus* species: 3–4 in *B. auleticus*, 11–12 and 15–16 in *B. brachyanthera*, and 12–13 in *B. catharticus* (Figure 1, Table 1).

The three *Bromus* species presented only metacentric and submetacentric chromosomes. Despite belonging to the same class, the chromosomes differed intra- and inter-

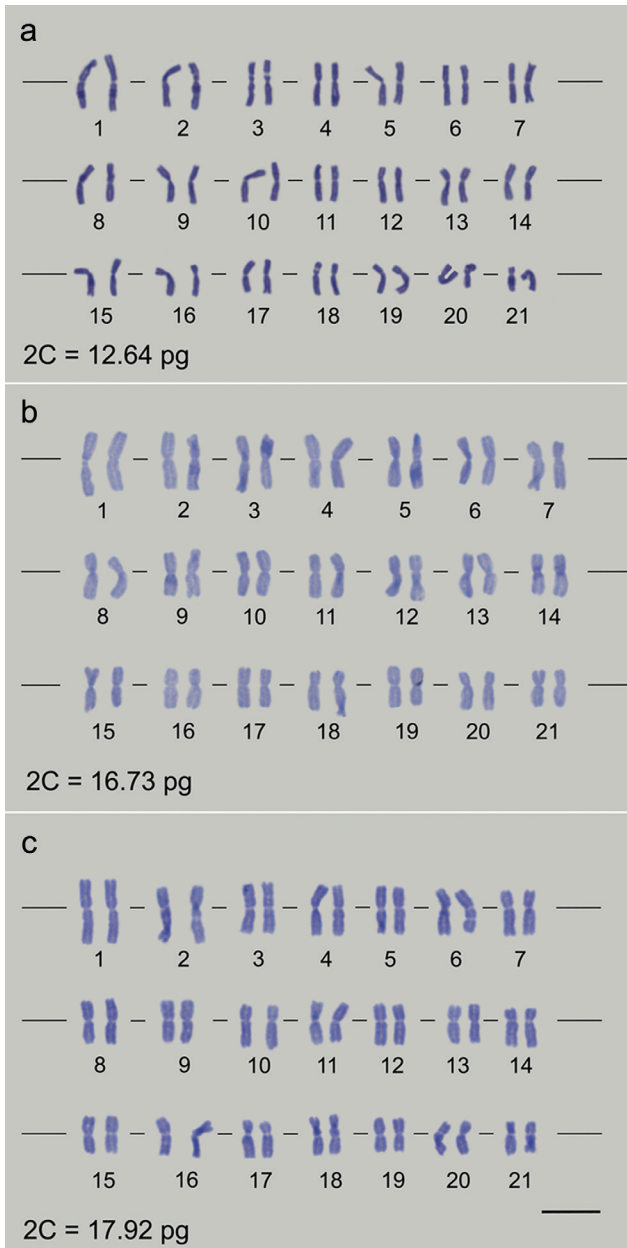


Figure 1. First karyograms of *Bromus* species, displaying $2n = 6x = 42$ chromosomes: **a** *B. catharticus* **b** *B. brachyanthera* and **c** *B. auleticus*. **a** *B. catharticus* displayed eleven metacentric (1, 4, 5, 7, 8, 9, 10, 11, 14, 17 and 20) and ten submetacentric chromosomes (2, 3, 6, 12, 13, 15, 16, 18, 19 and 21) **b** *B. brachyanthera* exhibited 13 metacentric (1, 2, 4, 5, 6, 9, 10, 13, 15, 16, 17, 19 and 21) and eight submetacentric chromosomes (3, 7, 8, 11, 12, 14, 18 and 20) **c** *B. auleticus* presented eleven metacentric (1, 3, 4, 5, 6, 8, 9, 11, 15, 18 and 19) and ten submetacentric chromosomes (2, 7, 10, 12, 13, 14, 16, 17, 20 and 21). Note the morphologically similar chromosomes: **a** 12–13 in *B. catharticus* **b** 11–12 and 15–16 in *B. brachyanthera*, and **c** 3–4 in *B. auleticus*. Bar = 5 μ m.

Table 1. Morphometry of the metaphasic chromosomes of *B. auleticus*, *B. brachyanthera* and *B. catharticus*.

Chrom.	<i>B. auleticus</i>						<i>B. brachyanthera</i>						<i>B. catharticus</i>					
	Total (μm)	Arms		r	Class	Size (%)	Total (μm)	Arms		r	Class	Size (%)	Total (μm)	Arms		r	Class	Size (%)
		Short	Long					Short	Long					Short	Long			
1	5.867	2.667	3.200	1.20	M	7.55	5.233	2.467	2.767	1.12	M	6.41	4.567	2.233	2.333	1.04	M	6.96
2	4.833	1.733	3.100	1.79	SM	6.22	4.767	2.133	2.633	1.23	M	5.84	3.833	1.500	2.333	1.56	SM	5.85
3	4.333	2.067	2.267	1.10	M	5.58	4.733	1.867	2.867	1.54	SM	5.80	3.800	1.500	2.300	1.53	SM	5.80
4	4.333	1.967	2.367	1.20	M	5.58	4.533	1.900	2.633	1.39	M	5.55	3.700	1.633	2.067	1.27	M	5.64
5	4.100	1.867	2.233	1.20	M	5.28	4.500	2.067	2.433	1.18	M	5.51	3.467	1.567	1.900	1.21	M	5.29
6	3.967	1.700	2.267	1.33	M	5.11	4.100	1.967	2.133	1.08	M	5.02	3.467	1.367	2.100	1.54	SM	5.29
7	3.900	1.433	2.467	1.72	SM	5.02	4.000	1.433	2.567	1.79	SM	4.90	3.400	1.667	1.733	1.04	M	5.19
8	3.900	1.667	2.233	1.34	M	5.02	3.933	1.500	2.433	1.62	SM	4.82	3.367	1.600	1.767	1.10	M	5.13
9	3.733	1.667	1.967	1.18	M	4.68	3.900	1.933	1.967	1.02	M	4.78	3.233	1.300	1.933	1.49	M	4.93
10	3.633	1.400	2.333	1.67	SM	4.80	3.867	1.833	2.033	1.11	M	4.74	3.167	1.567	1.600	1.02	M	4.83
11	3.600	1.633	1.967	1.20	M	4.63	3.800	1.467	2.333	1.59	SM	4.66	3.100	1.300	1.800	1.38	M	4.73
12	3.567	1.333	2.233	1.68	SM	4.59	3.800	1.467	2.333	1.59	SM	4.66	3.067	1.210	1.857	1.53	SM	4.68
13	3.333	1.300	2.033	1.56	SM	4.29	3.600	1.567	2.033	1.30	M	4.41	2.967	1.170	1.797	1.54	SM	4.52
14	3.333	1.100	2.233	2.03	SM	4.29	3.467	1.367	2.100	1.54	SM	4.25	2.967	1.267	1.700	1.34	M	4.52
15	3.333	1.567	1.767	1.13	M	4.29	3.433	1.533	1.900	1.24	M	4.21	2.800	1.033	1.767	1.71	SM	4.27
16	3.300	1.300	2.000	1.54	SM	4.25	3.433	1.500	1.933	1.29	M	4.21	2.733	0.867	1.867	2.15	SM	4.17
17	3.167	1.100	2.067	1.88	SM	4.08	3.400	1.533	1.867	1.22	M	4.17	2.733	1.300	1.433	1.10	M	4.17
18	3.000	1.467	1.533	1.05	M	3.86	3.400	1.200	2.200	1.83	SM	4.17	2.700	1.067	1.633	1.53	SM	4.12
19	2.933	1.300	1.633	1.26	M	3.78	3.300	1.500	1.800	1.20	M	4.04	2.367	0.800	1.567	1.96	SM	3.61
20	2.833	1.133	1.700	1.50	SM	3.65	3.267	1.033	2.233	2.16	SM	4.00	2.267	0.933	1.333	1.43	M	3.46
21	2.700	1.067	1.633	1.53	SM	3.47	3.167	1.300	1.867	1.44	M	3.88	1.867	0.600	1.267	2.11	SM	2.85
Sum	77.700	32.470	45.230	-	-	100.00	81.630	34.570	47.070	-	-	100.00	65.570	27.480	38.090	-	-	100.00

Chrom – chromosome; r – arm ratio (long/short); Size – % size in relation to sum of the mean values of total length; M – metacentric; SM – submetacentric; Sum – sum of the mean values.

specifically based on their morphology, which was characterized by occurrence of well-defined telomere and centromere portions and relatively low chromatin compaction level (Figure 1). As summarized in Table 1, the majority of the chromosomes could be distinguished by at least one morphometric parameter: total length, short- and long-arm length, ratio between arms, and/or relative chromosome size (%) in relation to the sum of the total length (Table 1). In addition, some chromosomes showed the secondary constriction in the interstitial region of the short arm, such as the chromosome 3 of *B. catharticus* (Figure 1a) or 18 of *B. auleticus* (Figure 1c).

Discussion

According to the chromosome number found here and the complement set $x = 7$ (Stebbins 1981), *B. auleticus*, *B. brachyanthera* and *B. catharticus* are hexaploid species ($2n = 6x = 42$). This result is in accordance with previous data reported for the three species (Schifino and Winge 1983, Naranjo 1985, Martinello and Schifino-Wittmann 2003). The genus *Bromus* originated in Eurasia, whereas the hexaploid species emerged in South America during the Pleistocene, from the subgenus *Ceratochloa* (Stebbins 1981). Thus, the chromosome number of the three species has remained conserved in relation to the ancestors, supporting Stebbins's hypothesis on the diversification of the *Bromus* species.

The mean nuclear 2C value divergences were corroborated by chromosome morphometry. *B. catharticus* clearly differed from the other species, being that its relatively small genome size correlated with the sum of the total chromosome length. Differently, for *B. auleticus* and *B. brachyanthera* this relation was not observed, as a result of the low compaction level of the chromatin in the latter species (Figure 1, Table 1). The large differences among nuclear 2C values for the same chromosome number suggests that the three species diverged through chromosomal rearrangements. In a study of hexaploid *Bromus* species, Klos et al. (2009) also reported interspecific variations ($2C = 12.72$ to 15.10 pg) in relation to the nuclear genome size. According to these authors, chromosomal changes occurred during the evolution of the hexaploid *Bromus* species, most likely through the gain or loss of highly repeated DNA sequences. Regarding this, karyotype and nuclear genome size should be considered together when comparing *Bromus* species.

Despite the similar morphology of some chromosome pairs (12–13 in *B. catharticus*, 11–12 and 15–16 in *B. brachyanthera*, and 3–4 in *B. auleticus*; Figure 1, Table 1), differences were found for most pairs of the three hexaploid *Bromus* species (Figure 1, Table 1). For the genus *Bromus*, Stebbins (1981) classified all species as allohexaploids. The allopolyploidy in *Bromus* was also found in *Bromus hordeaceus* L., 1753, which was classified as an allotetraploid (Ainouche et al. 1999). In that sense, Klos et al. (2009) highlighted that the *Bromus* section *Ceratochloa* includes a number of closely related allopolyploid species originated by three ancestors AABBCC.

Conclusion

The nuclear 2C value and karyotype characterization allowed differentiating the three *Bromus* species, thus contributing to the cytotaxonomy and evolutionary understanding in this taxon. As also demonstrated by other authors, these data provide insights about the evolutionary process and diversification of the polyploid *Bromus* species.

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