

Pollen viability and meiotic abnormalities in brome grasses (*Bromus* L., Section *Ceratochloa*) from Argentina

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Abstract: *Bromus* Section *Ceratochloa* includes temperate forage species. South American species form a morphologically very variable hexaploid complex ($2n = 6x = 42$), with a controversial taxonomic classification. Reproductive biology has been studied in several of them, but the number of analyzed plants has seldom been reported. Further investigations might clarify breeding relations and the adequacy of current classifications. Pollen viability and size, which can provide evidence of spontaneous hybridization, were studied in seven accessions (six plants/accession) classified as *B. catharticus* var. *catharticus*, var. *elata*, and var. *rupestris*; *B. parodii*; *B. bonariensis*; *B. lithobius*; and *B. coloratus*. Variability was detected among accessions for average pollen viability (54%–82%) and pollen size (15.8%–26.2% $<n$ pollen, 44.8%–72.2% n (normal), and 12.0%–29.0% $>n$). Meiotic analyses were performed in a sample of genotypes from five accessions with $<70\%$ pollen viability and/or variability in pollen size. Normal and abnormal meiocytes (11.9%–44.5%) were recorded at various meiotic stages in one randomly taken genotype from each sample. Since these cleistogamous species can produce chasmogamous flowers, the analyzed accessions, conserved as "species", are apparently of hybrid origin. Segregation in hybrid populations could add to the confusion in taxonomy, affecting both conservation of genetic diversity and efficiency of parental selection in breeding.

Key words: Forage grasses, pollen size, pollen viability, meiosis, hybrids

1. Introduction

Genus *Bromus* L., which belongs to the family Poaceae (Gramineae), contains 100 to 400 species (Pavlick and Anderton, 2007), defined on the basis of morphological phenotypes (typological species). These species form a polyploid series with $2n = 2x, 4x, 6x, 8x, 10x,$ and $12x$ ($x = 7$) somatic chromosomes (Armstrong, 1991). Some of them are annual, while others are either biennial or perennial, caespitose, or rhizomatous (Pavlick and Anderton, 2007), autogamous or allogamous (Armstrong, 1991). They are widely distributed in temperate and mountainous regions of Asia, Europe, Africa, and America (Williams et al., 2011; Gutiérrez and Pensiero, 2012).

Typological species, being human constructions, are subjected to periodical revisions. In the most accepted classification (Smith, 1985, in Williams et al., 2011), *Bromus* species are grouped into seven sections. One of these sections, *Ceratochloa*, is agronomically very important because it contains species of forage value.

Hybridization and structural chromosome differentiation have played a major role in polyploid

evolution in section *Ceratochloa* (Stebbins, 1956). North American species have $2n = 8x$ and $2n = 12x$ chromosomes, whereas South American species, which are closely related and most of them $2n = 6x$, form a hexaploid complex (Williams et al., 2011). According to Stebbins (1956), these species have an allopolyploid origin: extinct $2x$ and $4x$ species might have been involved in the formation of the hexaploids (genomic formula $A_1A_1B_1B_1B_2B_2$), whereas the $8x$ North American species (genomic formula $A_1A_1B_1B_1B_2B_2LL$) probably originated from hybridization between $6x$ species of section *Ceratochloa* and $2x$ South American species of section *Pnigma* Dumort. (= *Bromopsis* Dumort.), which provided the long (L) chromosomes. Stebbins (1956) also proposed that the $12x$ species could be the product of hybridization at the hexaploid level between species of section *Ceratochloa* and *B. berterioanus* Colla (= *B. trinii* E.Desv.) of section *Neobromus* (Shear.) Hitchcock. More recently, Klos et al. (2009) demonstrated that the A_1 , B_1 , and B_2 genomes are almost undistinguishable.

Variation in morphological phenotypes in the hexaploid complex is large; therefore, the taxonomy is

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controversial. The complex was originally subdivided into a number of species, but after further investigations using morphological and/or molecular markers, many species were grouped into a hypervariable one (Planchuelo, 1991, 2006; Peterson and Planchuelo, 1998; Massa, 2004; Williams et al., 2011). In this regard, Williams et al. (2011, p. 24) pointed out that “Hybridization is rife in this section, making species boundaries obscure and the taxonomy very difficult”. However, accessions in germplasm banks are generally assigned (and conserved under) the category of “species” (see an example at <http://www.ars-grin.gov/npgs/collections.html>).

Aspects of the reproductive behavior of *Bromus* species (commonly known as brome grasses) have been studied by several authors. Flower type, mode of reproduction, seed formation, and viability in populations of *Bromus catharticus* var. *catharticus* were reported by Ragonese and Marcó (1941, 1943), Langer and Wilson (1965), Naranjo (1992), and Wolff et al. (1996, 2001). There are also reports on pollen viability and meiotic behavior in plant samples of natural populations of *Bromus catharticus* var. *catharticus* and artificial hybrids involving this botanical variety, and of other species or botanical varieties of the same section from Argentina (Stebbins and Tobgy, 1944; Hall, 1955; Naranjo, 1992). However, information on the number of plants analyzed in those studies is either lacking or scarce. Thus, it cannot be ascertained if the reported results represent the reproductive behavior of natural populations or only the behavior of the particular genotypes that were subjected to analysis. Further reproductive biology studies may contribute to shedding light on the breeding relations among members of the hexaploid complex, and this information would contribute to their classification, ex situ conservation, and utilization of the available genetic diversity for breeding and other applied purposes.

As a first step in the study of the reproductive biology of a plant group, the estimation of pollen viability and size—and the observation of the underlying meiotic events leading to male gametophyte formation— can facilitate the detection of hybrids in both natural populations and their samples (accessions in germplasm banks). This information is of relevance for planning germplasm collections and sampling strategies. Herein, we report the results of pollen viability and pollen size analyses in seven accessions of South American populations of *Bromus* (section *Ceratochloa*) and the meiotic behavior of genotypes with low pollen viability and/or variability in size. We also discuss their possible origin and consequences of the findings in accessions classified as “species”, for both conservation and utilization of the natural genetic diversity.

2. Materials and methods

The plant materials were individual plants from seven accessions of *Bromus* L., section *Ceratochloa* (Poaceae Barnhart), one from Chile and six from Argentina, cultivated as part of a field trial at the College of Agrarian Sciences (FCA), National University of Mar del Plata (UNMDP)/Experimental Agricultural Station “Domingo Pasquale”, National Institute of Agricultural Technology (INTA), Balcarce, Argentina (37°45'S, 58°18'W, 130 m a.s.l.). These accessions had been previously classified by SI Alonso and ML Echeverría (FCA, UNMDP) into five taxonomic species, one of them with three botanical varieties: *Bromus catharticus* Vahl. var. *catharticus*, var. *rupestris* (Speg.) Planchuelo & Peterson, and var. *elata* (Desv.) Planchuelo; *B. bonariensis* Parodi & Cámara; *B. parodii* Covas & Itria, *B. coloratus* Steud.; and *B. lithobius* Trin. (Table 1). Only the *B. lithobius* accession was derived from seeds that had been originally collected in nature.

Table 1. *Bromus* species and botanical varieties, accessions, and geographic coordinates of the originally sampled populations.

Plant material		Abbr.	Geographic coordinates		Province/region and country
Species/botanical variety	Accession		Lat. (S)	Long. (W)	
<i>B. bonariensis</i>	BAL 2008197	Bbon197	38°3'58.2"	57°32'41.5"	Buenos Aires, Argentina
<i>B. catharticus</i> var. <i>catharticus</i>	BAL 2008195	Bcat195	37°44'40.2"	57°25'9.1"	Buenos Aires, Argentina
var. <i>rupestris</i>	BAL 2008201	Brup201	36°34'5.5"	61°44'51.1"	Buenos Aires, Argentina
var. <i>elata</i>	BG 403	Bela403	45°23'57.4"	72°41'19.9"	Aysén, Chile
<i>B. coloratus</i>	BG 252	Bcol252	38°49'35.5"	68°4'1.9"	Río Negro, Argentina
<i>B. lithobius</i>	BAL 990205	Blit205	40°09'1.3"	71°37'28.2"	Neuquén, Argentina
<i>B. parodii</i>	BAL 2008193	Bpar193	37°50'31.5"	57°30'4.2"	Buenos Aires, Argentina

Seeds of the remaining six accessions corresponded to the first ex situ multiplication, which had been carried out in the experimental field without isolation measures. Chromosome numbers ($2n = 6x = 42$) of the original accessions are available at the Germplasm Bank of Potato and Forages, INTA, Balcarce.

2.1. Pollen viability

Pollen viability was indirectly estimated under an optical microscope by staining. To that end, at bloom, inflorescences from six plants (genotypes) per accession were taken to the laboratory for pollen extraction. Fresh pollen was sprinkled on glass slides and stained with acetocarmine solution (0.2 g carmine, 45 mL glacial acetic acid, and 55 mL distilled water) for observation. Around 300 pollen grains were observed per plant. Pollen grains that were fully stained, plump, and round and regular in shape were considered viable, whereas those that were poorly (or not) stained and/or irregularly shaped were considered nonviable. The percentage of pollen viability per plant was calculated by considering the number of viable pollen grains over the total number of observed pollen grains.

2.2. Pollen grain size

Pollen grain diameter was measured after staining under an optical microscope. Microphotographs of each sample were taken with an Olympus Q-Color 3 digital camera (Olympus America Inc., Center Valley, PA, USA) and processed with the application software for image analysis MicroMeasure 3.3 (Reeves and Tear, 2000). Pollen grains (300 per genotype) were classified into three categories (<n, n = normal, and >n) according to the ratio between their individual diameters and the average diameter of the sample. Pollen grains with diameter ratios below 0.9, between 0.9 and 1, and above 1 were considered <n, n, and >n, respectively. The frequency of pollen grains in each of the three categories was calculated on an individual genotype basis.

2.3. Meiotic analyses

Meiotic analyses were carried out in a sample of genotypes with low pollen viability (<70%) and/or variability in pollen size, detected in each of the following accessions: Bbon197, Brup201, Bcat195, Bpar193, and Bcol252. Flower buds, extracted at the boot stage, were fixed in a 96% ethanol:acetic acid solution (3:1, v/v) for 24 h and then transferred to and conserved in 70% ethanol until use. One anther from each bud was placed on an acetocarmine drop on a glass slide, and meiocytes were released with the help of a needle. Coverslips were placed on the preparations for observation under a light microscope, starting the analysis from the more advanced stages and working backwards to earlier ones. Normal and abnormal meiocytes at various meiotic stages were recorded in one randomly taken

genotype from the meiotically analyzed sample of each accession.

3. Results

3.1. Pollen viability

The percentage of pollen viability was variable among accessions and among genotypes within accessions. Bpar193 had the highest mean value ($\bar{x} = 82\%$), whereas Bcat195 had the lowest ($\bar{x} = 54\%$) but exhibited the largest intrapopulation range (from 25% to 87%) (Figure 1).

3.2. Pollen size

Variability was observed for both pollen grain size and pollen grain frequency in each size category (<n, n, >n) in the seven accessions (Figure 2). Bpar193 had the highest percentage of normal size pollen (72.2%), whereas Bela403 had the lowest (44.8%). The percentage of abnormal size pollen grains was similar in the <n and >n size categories (Table 2).

3.3. Meiotic analyses

Meiotic abnormalities were detected in flowers buds of the sample of plants analyzed in each of the five accessions. In the randomly taken genotypes in which normal and abnormal meiocytes were recorded, the abnormalities observed were: out of plate chromosomes in metaphase I and II; lagging chromosomes in anaphase I and II and in telophase I and II; chromosome asynchrony in anaphase II and in telophase I and II; asynchrony between the two second meiotic division cells; abnormal disposition of the division planes in metaphase II; and dyads, triads, and abnormal tetrads at the tetrad stage (see a few examples in Figures 3a–3j). The percentage of abnormal meiocytes varied among genotypes. Genotype “Bbon197 III-19” had the highest percentage of abnormalities (44.7%), whereas “Bcol252 III-1” had the lowest (11.9%) (Table 3).

4. Discussion

4.1. Pollen viability

The seven studied accessions had been assigned to the category of “species” for ex situ conservation and multiplication. Notwithstanding this, we observed variability in pollen viability and size not only among accessions but also among plants within accessions, particularly in accessions Bcat195 and Bcol252. Similarly, for samples of natural populations of four out of the seven species/botanical varieties analyzed by us, Naranjo (1992) reported variability for pollen viability, with values above 75.0%. However, this author did not mention the number of analyzed genotypes, except for one population of *B. parodii* (one plant) and another population of *B. bonariensis* (two plants), which contrasts with the six plants per accession analyzed in our study. Additionally, in artificial interspecific hybrids, Naranjo (1992) reported pollen viability values of

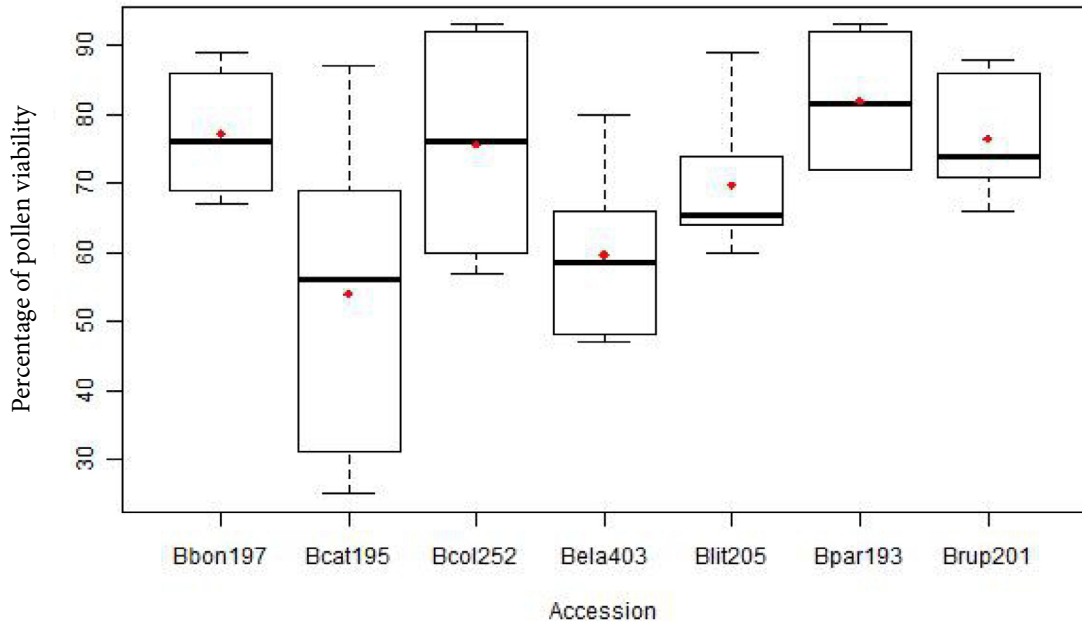


Figure 1. Percentage of pollen viability in seven accessions of five species of *Bromus*, one of them with three botanical varieties. Box = 50% of the central data; highlighted line = median; red point = mean; bottom and top lines = minimum and maximum.

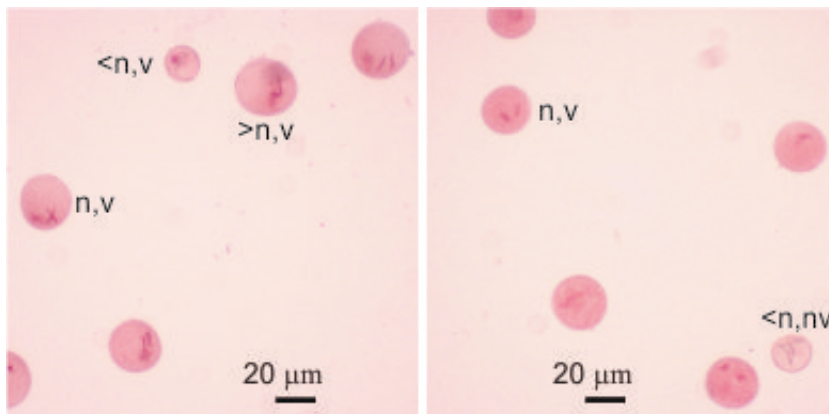


Figure 2. Variability in pollen size and viability in two individuals of *Bromus* (section *Ceratochloa*) from southeastern Buenos Aires Province. n: Normal size, v: viable, nv: nonviable.

Table 2. Average percentage of pollen grains classified in the <n, n, and >n size categories per accession. In parentheses, maximum and minimum individual plant values.

Accession	Pollen size category (%)		
	<n	n	>n
Bcat195	23.2 (19.5–32.4)	55.6 (42.0–62.3)	21.2 (16.9–25.6)
Bbon197	19.2 (15.8–23.1)	61.0 (53.2–68.4)	19.8 (15.7–23.7)
Brup201	23.7 (12.0–32.4)	54.0 (36.9–73.1)	22.3 (14.9–30.7)
Bcol252	23.5 (6.8–37.0)	52.0 (26.1–87.4)	23.5 (5.8–37.5)
Blit205	20.7 (15.1–27.2)	58.1 (43.9–73.0)	21.2 (11.7–28.9)
Bela403	26.2 (22.0–31.0)	44.8 (37.8–52.6)	29.0 (25.0–32.0)
Bpar193	15.8 (6.6–26.2)	72.2 (44.4–92.1)	12.0 (1.3–29.5)

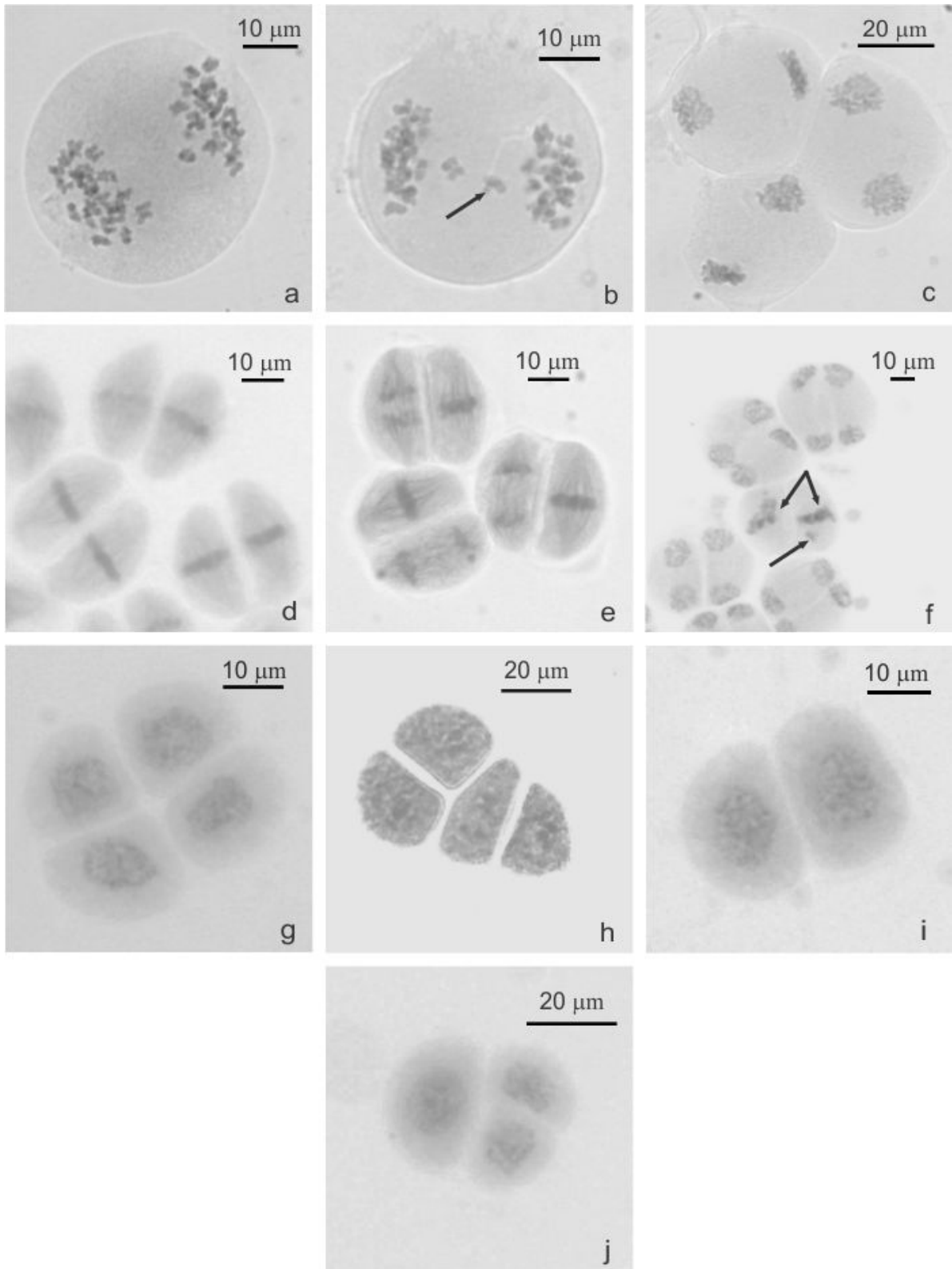


Figure 3. Meiosis in plants with low pollen viability. Normal anaphase I (a); lagging chromosomes in late anaphase I (arrow) (b); chromosome asynchrony in telophase I (c); metaphase II: normal (d), asynchrony in the two cells of the meiocyte (e); abnormal disposition of the division planes and out-of-plate chromosomes (arrows) (f); normal tetrad (g); abnormal tetrad (h); dyad (i); triad (j).

Table 3. Number of normal (N) and abnormal (A) meicytes at various meiotic stages in five genotypes of four *Bromus* species, one of them with two botanical varieties. M: Metaphase, A: anaphase, T: telophase, n: number of observed cells.

Genotype	Meiosis I						Meiosis II						Tetrad stage			n	Abnormal meicytes (%)		
	M		A		T		M		A		T		Tetrad	Dyad	Triad				
	N	A	N	A	N	A	N	A	N	A	N	A	N	A					
Bbon197 III-19					25	27							66	27	9	10	164	44.5	
Brup201 I-11					65	3		4				15	4	38	12	9	1	151	21.9
Bcat195 I-10	62	4			25	15												106	17.9
Bpar193 II-2	52		54	2	1		20	15	12	1	5		44	2	6	2		216	13
Bcol252 III-1													52	7	0	0		59	11.9

2.4% up to 51.0%, which are similar to some of the values recorded by us, although we worked with accessions ex situ conserved and multiplied as “species”.

4.2. Pollen size and meiotic behavior

There is no information in the literature about pollen grain size in brome grasses, but in addition to variability for pollen viability, we detected variability for pollen size in each of the analyzed genotypes. In this regard, abnormalities in chromosome pairing and segregation in higher plants can lead to the formation of $<n$ and $>n$ pollen. Moreover, gametes or gametophytes with sporophytic chromosome numbers ($2n$ gametes) can be formed both spontaneously, by random events in mitosis or meiosis, or by heritable cytological mechanisms in meiosis (Carputo et al., 2006).

In the meiotic analysis, we observed abnormalities at various stages in all accessions that might have a heritable base. The most relevant for their genetic consequences were asynchrony in chromosome movement at metaphase-anaphase II in either one or both cells of the meicytes, plus dyads and triads at the tetrad stage, in three out of the four analyzed genotypes. Dyads would lead to the formation of two $2n$ pollen grains and triads to one $2n$ and two n pollen grains; both abnormalities would account for the $>n$ pollen grains observed in plants of the seven accessions, regardless of their pollen viability. Thus, we propose that these abnormalities have a genetic base and, therefore, are not the result of random events at meiosis.

Genetically controlled $2n$ gamete formation in meiosis is regarded as one of the most frequent mechanisms involved in sexual polyploidization, in contrast to asexual polyploidization, in which the uniting gametes are one or both numerically unreduced but due to random pre- or postmeiotic chromosome duplication events in mitosis. In sexual polyploidization, the transmission of intra- and interlocus interactions present in the parental genotypes to the progeny and the extent of heterotic responses in the latter can reach their maximum (Carputo et al., 2006). We consider that the production of $>n$ pollen grains observed

in this study can be associated with sexual polyploidization events in the origin of the polyploid series in *Bromus* section *Ceratochloa*, described by Stebbins (1956), and that the genetic consequences of this mode of polyploidization have been one of the factors leading to their successful establishment in nature.

The perpetuation of a sexually reproducing species would be dependent, in the first place, on its fertility, which results from regular chromosome pairing and segregation in meiosis. Stebbins and Tobgy (1944) reported regular bivalent pairing at metaphase I of meiosis in various accessions of *Bromus* (Sect. *Ceratochloa*) although, as previously pointed out, they did not mention the number of plants analyzed per population. In artificial $6x$ interspecific hybrids, Stebbins and Tobgy (1944) and Hall (1955) reported bivalent pairing, but also the presence of univalents, trivalents, and tetravalents in low percentages, which is indicative of chromosome homologies between the parental species. In this regard, Stebbins (1945) mentioned that high pollen sterility in higher plants in which in meiotic pairing is regular could be caused by some degree of genome differentiation or “cryptic structural hybridity”.

The analyzed accessions have cleistogamous flowers that are self-pollinated. However, during part of their growing cycle they can produce chasmogamous flowers, which provide opportunities for cross-pollination (Leofanti et al., 2013). Some of the abnormalities observed in meiosis, such as out-of-plate and lagging chromosomes, which reduce male fertility due to the formation of genetically unbalanced gametophytes, would indicate that these accessions are of a likely hybrid origin, as a result of gene flow during the chasmogamous period in areas of sympatry, either in nature or in the experimental field in which the first ex situ multiplication was carried out. In this regard, *B. brevis* is sympatric with *B. catharticus* in temperate humid regions; moreover, spontaneous *B. catharticus*-*B. brevis* hybrids have been reported (Itria, 1958), revealing

that the two typological species are sexually compatible, even though they have been mentioned in the literature as having different ecological preferences.

Morphological and genetic variability is expected in natural *Bromus* populations because cleistogamy is not strict (Leofanti et al., 2013); thus, hybridization is possible in areas of sympatry. Hybrids can be fertile if chromosome differentiation between the parents is not high or if it is absent. Notwithstanding this, germplasm collection and conservation activities in brome grasses are carried out by considering the taxonomic classification based on morphological phenotypes, regardless of breeding relations. Populations and accessions are usually classified as pure

species even though natural populations with at least a percentage of allogamy could consist of pure "species", F₁ hybrids, and/or advanced segregating generations. In our opinion, it is necessary to consider this phenomenon before assigning specific categories to natural populations or their samples, due to the consequences in the conservation of the available genetic diversity at the threat of global climate change, and for parental selection in breeding.

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