

Genome elimination during microsporogenesis in two pentaploid accessions of *Brachiaria decumbens* (Poaceae)

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ABSTRACT. Polyploidy is a prominent and significant force in plant evolution, taking place since ancient times and continuing until today. Recent cytogenetic studies in the genus *Brachiaria* using germplasm collected from wild African savannas in the 1980s revealed that most species and accessions within species are polyploid. Diploid, tetraploid, and pentaploid accessions have been found. We found asynchronous meiosis during microsporogenesis, followed by genome elimination, in two pentaploid ($2n = 5x = 45$) accessions (D53 and D71) of a hardy, invasive pasture grass, introduced from Africa to Brazil, *Brachiaria decumbens*. In these accessions, chromosomes paired as 18 bivalents and nine univalents during diakinesis, suggesting that these accessions resulted from a recent event of natural hybridization. The lack of chromosome associations in the genomes suggests that these accessions resulted from hybridization between two genotypes that are not closely related, with low genome affinity and with different meiotic rhythms. This supposition is reinforced by the meiotic behavior of the nine univalents, which were always laggard in relation to the other chromosomes and eliminated as micronuclei

in microspores. The behavior of these accessions, which have an odd level of ploidy and confirmed genome elimination, supports the general assumption that a polyploid accession can undergo a new event of polyploidization by natural hybridization (neopolyploidization). This evidence for natural hybridization in *Brachiaria* shows that this is a wild genus in an ongoing evolutionary process.

Key words: *Brachiaria decumbens*; Natural hybridization; Polyploidy; Genome elimination; Microsporogenesis; Evolution

INTRODUCTION

Polyploidy is a prominent and significant force in plant evolution, occurring since ancient and continuing up to contemporary times, and with profound effects ranging from molecular to ecological (Adams and Wendel, 2005). The more recent advent of whole genome sequencing and comparative mapping studies have shown that some species traditionally considered as classical diploids are actually ancient or paleopolyploids (Leitch and Bennett, 2004). According to Soltis (2005) probably all angiosperms are polyploid to some extent.

Traditionally, polyploidy refers to either duplication of a single genome (autopolyploidy) or to the combination of two or more differentiated genomes (allopolyploidy) (Wendel and Doyle, 2005). Some evidence indicates that both autopolyploidy and allopolyploidy are common in nature, and that the latter probably predominates (Ramsey and Schemske, 1998; Soltis et al., 2004; Wendel and Doyle, 2005).

Previous studies in the genus *Brachiaria* revealed that most species and accessions within the species are polyploid (Pritchard 1967; Nassar 1977; Ndikumana 1985; Basappa et al. 1987; Valle and Savidan, 1996; Utsunomiya et al., 2005; Mendes-Bonato et al., 2002, 2006a; Pagliarini et al., 2008; Risso-Pascotto et al., 2003, 2006a, 2009a,b). Among the polyploid accessions there is evidence that some of them evolved from autopolyploidy (Mendes-Bonato et al., 2002) and others from allopolyploidy (Mendes et al., 2006; Boldrini et al., 2009a,b, 2010). Evidence of recent natural hybridizations has been found in some accessions with odd levels of ploidy, such as *Brachiaria brizantha* (Mendes et al., 2006; Risso-Pascotto et al., 2006b) and in *B. humidicola* (Boldrini et al., 2009a,b, 2010). In these accessions, the evidence of recent hybridization was provided by cytological phenomena, such as i) the formation of independent metaphase plates between genomes; ii) the organization of two nucleoli in hybrid meiocytes, and iii) genome elimination by asynchronous meiosis. These phenomena reveal that the parental genomes are not yet adapted to work in the same cytoplasm.

Brachiaria decumbens was the first species of this genus introduced to Latin America in the early 1950s (Serrão and Simão Neto, 1971) and one of its cultivars, 'Basilisk', is the most widely planted forage species for pastures in the tropics even today (Valle and Pagliarini, 2009). This cultivar is widely used in Brazil because it is very productive, adapts well to acid soils, is easy to manage and readily establishes from seed. It is, however, highly susceptible to a group of sucking insects called "spittlebugs" (Homoptera: Cercopidae) (Valério and Nakano, 1988), and has been associated with photosensitization in cattle in Brazil (Barbosa et al., 2006). Nevertheless, it is an excellent forage that presents several agronomic attributes thus justifying a breeding program to overcome these limitations.

Cytological studies in the germplasm collection of this species carried out to support the breeding program revealed the presence of diploid, tetraploid and pentaploid accessions. In this paper, we report the occurrence of asynchronous meiosis during microsporogenesis in the pentaploid accessions of this germplasm collection followed by genome elimination.

MATERIAL AND METHODS

Plant material

Both accessions of *B. decumbens* under analysis, available at Embrapa Beef Cattle Research Center (Campo Grande, MS, Brazil) were collected in wild African savannas in the 1980s by the International Center for Tropical Agriculture (CIAT, Colombia), transferred to Embrapa Genetic Resources and Biotechnology (Brazil), and after quarantine, to Embrapa Beef Cattle, Campo Grande, MS. In Embrapa Beef Cattle, these accessions are maintained in the field since 1988, in plots of 16 plants, where site characteristics of cultivation are: tropical wet climate, group A: tropical humid savanna; average annual precipitation = 1526 mm; average temperature = 22°C; altitude 520 m; latitude = 20°28' S; longitude = 55°40' W; poor dark red Latossol soil composed of 59% sand, 8% silt and 33% clay; pH = 4.2.

Cytological analysis

Inflorescences for meiotic studies were collected from individual plants growing in the field, immersed in a solution containing 95% ethanol, chloroform and propionic acid (6:3:2, v/v) for 24 h and stored in 70% ethanol at 4°C. Anthers containing cells in meiosis were isolated from the flowers and slides with cell spreads that were obtained after squashing and staining with 0.5% propionic carmine solution. A total of 1749 pollen mother cells were analyzed under light microscopy for D53 and 1223 for D71. Images were obtained using Kodak Imagemink - HQ, ISO 25 black and white film.

The mode of reproduction of these accessions was previously determined (Valle and Savindan, 1996) under interference contrast microscopy on methylsalicylate-cleared ovaries (Young et al., 1979). Pollen fertility and successful development was estimated in mature pollen grains.

RESULTS AND DISCUSSION

Chromosome counting on slides containing microsporocytes at diakinesis and in well-spread anaphases I revealed that D53 and D71 were pentaploids, derived from $x = 9$, thus with $2n = 5x = 45$ chromosomes. Accessions with odd levels of ploidy are not common in the genus *Brachiaria* and this is the first report of pentaploidy in *B. decumbens*. Another few pentaploids have also been recorded in *B. brizantha* (Mendes-Bonato et al., 2002; Mendes et al., 2006; Risso-Pascotto et al., 2003, 2006b) and in *B. humidicola* (Boldrini et al., 2009a,b, 2010).

In the accessions here analyzed, chromosomes paired preferentially as bivalents (18 II) and univalents (9) in diakinesis (Figure 1a). The presence of the two sets of chromosome arrangements suggests that they are most likely originated through a recent event of natural hybridization with one of the progenitors contributing with the doubled genome (18 bivalents) while the other, with only one genome (9 univalents). Polyploidy is predominant in the genus

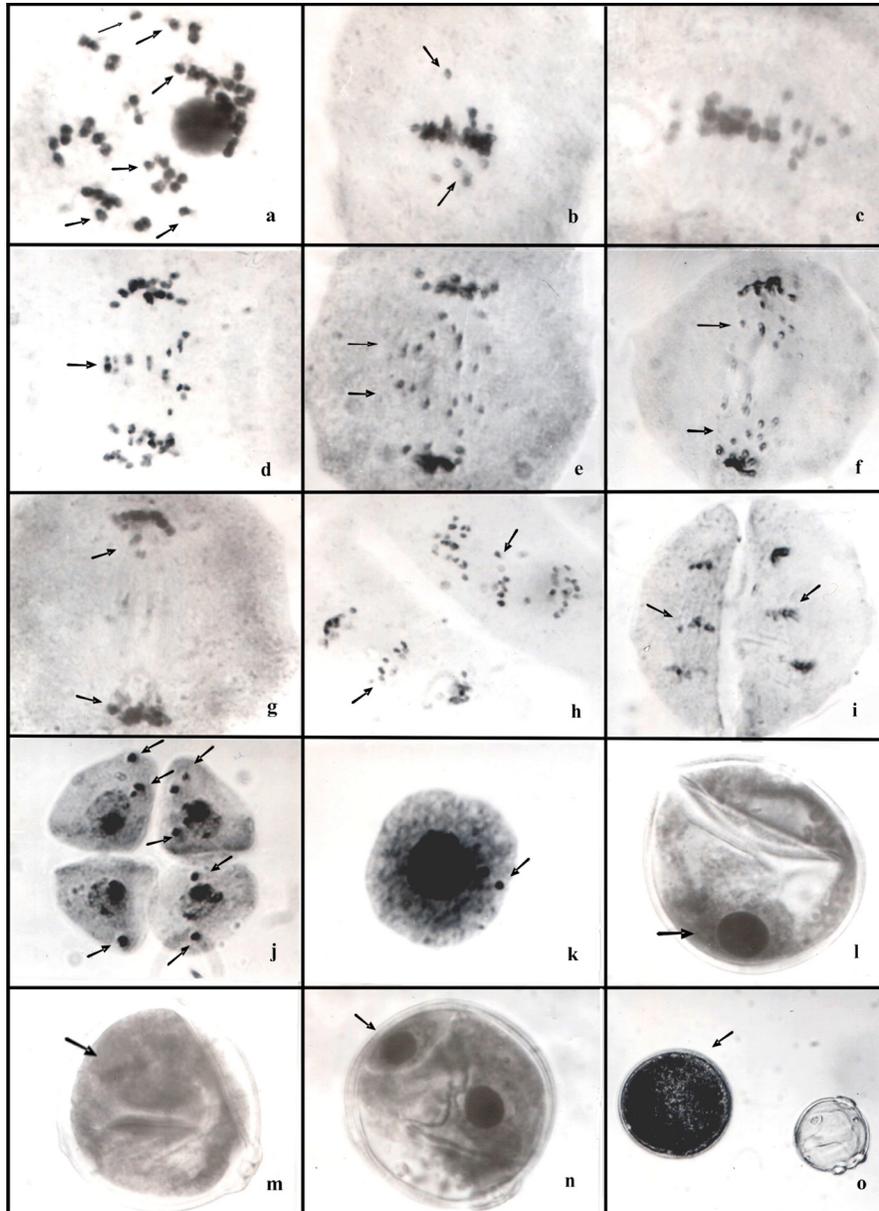


Figure 1. Aspects of the meiotic behavior of D53 and D71. **a.** Meioocyte in diakinesis showing 18 bivalents and the presence of univalents. **b,c.** Metaphase I with some univalents not congregated at metaphase plate. **d.** Late anaphase I for the major genome and metaphase I for the nine univalents. **e,f.** Late anaphase I for the major genome and anaphase I for the univalents that underwent sister chromatid segregation. **g.** Telophase I for the major genome and some micronuclei originated by the laggard chromatids. **h,i.** Anaphase II for the major genome and metaphase II for the nine chromatids. **j.** Tetrad with micronuclei in all microspores. **k.** Released microspore with micronuclei. **l.** Microspore with polarized nuclei. **m.** Microspore in metaphase of the first pollen mitosis. **n.** Pollen grain with both generative and vegetative cells. **o.** Stained fertile and unstained sterile pollen grains (magnification 400X).

Brachiaria and is correlated to apomixis (Valle and Savidan, 1996; Valle and Pagliarini, 2009) and the role of unreduced (male and female) gametes in the phenomenon is unquestionable. The formation of unreduced male gametes, originated by different mechanisms of abnormal cytokinesis, has been reported in *B. brizantha* (Risso-Pascotto et al., 2003), *B. nigropedata* (Utsunomiya et al., 2005), *B. jubata* (Mendes-Bonato et al., 2006a), and *B. humidicola* (Boldrini et al., 2006; Gallo et al., 2007; Calisto et al., 2008). In the accessions under analysis, one progenitor, probably apomictic contributed with an unreduced gamete ($2n = 36$) and the maternal contribution derived from a sexual diploid plant, with a reduced gamete ($N = 9$).

Additionally, the absence of pairing among the sets suggests that the potential genome donors are two species without genome affinity, i.e., not closely related and with different meiotic rhythm. This assumption is reinforced by the observation of the different timing of the bivalent and the univalent sets during the meiotic process. In metaphase I, the nine univalents reach the plate after the 18 bivalents (Figure 1b,c). In anaphase I, while the 18 bivalents were segregating towards the poles, the 9 congregated univalents organized their own metaphase plate (Figure 1d). When the 18 chromosomes reached the poles, the 9 univalents underwent chromatid segregation and initiated the migration to the poles (Figure 1e,f). However, only some of the chromatids reached the poles in time to be included in the telophase nuclei, leading to micronuclei formation (Figure 1g). During the second division, similar behavior was observed, with 9 laggard chromatids present in each metaphase II (Figure 1h,i). Since they underwent segregation during anaphase I, the segregation in anaphase II was also unequal. The chromatids formed different numbers of micronuclei in telophase II (Figure 1j). Micronuclei remained present within released microspores (Figure 1k). The percentage of abnormal tetrads was very high, reaching 100% in D53 and 97.9% in D71 and the overall abnormality frequencies for each accession are presented in Table 1.

Table 1. Number of cells analyzed during meiosis and the percentage of abnormal cells observed in each meiotic phase in the *Brachiaria decumbens* accessions D53 and D71.

Phases	D53		D71		Abnormalities
	No. of PMCs	No. of abnormal PMCs (%)	No. of PMCs	No. of abnormal PMCs (%)	
Diakinesis	30	30 (100.0%)	21	21 (100.0%)	Nine univalents
Metaphase I	318	12 (3.8%)	293	88 (30.3%)	Nine univalents outside plate
Anaphase I	170	142 (87.6%)	129	75 (58.1%)	Nine laggard univalents
Telophase I	74	50 (67.5%)	110	54 (49.0%)	Micronuclei in both poles
Prophase II	138	67 (48.5%)	209	59 (28.2%)	Micronuclei in both poles
Metaphase II	168	28 (16.6%)	184	44 (23.9%)	Nine chromatids outside plate
Anaphase II	142	140 (98.5%)	59	58 (96.6%)	Nine laggard chromatids
Telophase II	256	248 (96.8%)	90	82 (91.1%)	Micronuclei in both poles
Tetrad	483	483 (100.0%)	248	243 (97.9%)	Micronuclei in all microspores
Total	1779		1244		

PMCs = pollen mother cells.

The meiotic behavior described for D53 and D71 is also found in other polyploid accessions of *Brachiaria* with odd levels of ploidy, where one genome derived from $x = 9$ in *B. brizantha* (Mendes et al., 2006; Risso-Pascotto et al., 2006b) or derived from $x = 6$ in *B. humidicola* (Boldrini et al., 2009a,b, 2010) was eliminated in micronuclei by asynchrony during meiosis.

Polyploidy is regarded as the major mechanism of adaptation and speciation in plants (Ramsey and Schemske, 2002). Several studies, including molecular research (Ramsey and Schemske, 2002; Soltis, 2005; Soltis et al., 2003, 2007), have shown that recurrent polyploidy

is the rule, not the exception. According to Soltis (2005) these studies indicate that probably all angiosperms, and maybe all plants, are polyploid to some extent. Polyploidy is common in the genus *Brachiaria*, and tetraploidy prevails (Valle and Savidan, 1996; Penteadó et al., 2000; Utsunomiya et al., 2005; Mendes-Bonato et al., 2002, 2006a; Pagliarini et al., 2008; Risso-Pascotto et al., 2003, 2006a, 2009a,b; Boldrini et al., 2009a,b, 2010). It is estimated that most flowering plants are descendants of polyploid ancestors (Masterson, 1994). Several polyploids were formed in the past by events of genome duplication that occurred many millions of years ago (paleopolyploids), but polyploidy is an active and ongoing process (Adams and Wendel, 2005). The present accessions, and others recorded in *Brachiaria* with odd level of ploidy and genome elimination (Mendes et al., 2006; Risso-Pascotto et al., 2006b; Boldrini et al., 2009a,b, 2010) reinforce this assumption, showing that a polyploid accession can undergo a new event of polyploidization by natural hybridization (neopolyploidization). Abbott and Lowe (2004) maintained that the discovery of a new polyploid species in the wild soon after its origin presents an excellent opportunity to examine numerous phenomena concerning polyploid speciation and evolution. Although that is logical, such species have, regrettably, not been explored for that purpose. At least five angiosperm species are known to have originated via allopolyploidy in recent times, i.e., within approximately the past 150 years (see Abbott and Lowe, 2004). The evidence of natural hybridization in *Brachiaria*, originating accessions with odd levels of ploidy, illustrates that this is a wild genus in a continuous evolutionary process.

Relatively little is known about the genetic and functional consequences of uniting two divergent genetic systems into a common nucleus in only one parental cytoplasm (Wendel and Doyle, 2005). A critical period in this process is during and immediately after allopolyploid formation, when two distinct genomes are first brought into contact, thereby requiring a diverse array of accommodation (Liu et al., 2001). In the accessions D53 and D71, and in the other cases of hybridization followed by a genome elimination in *Brachiaria* (Mendes et al., 2006; Risso-Pascotto et al., 2006b; Boldrini et al., 2009a,b, 2010) it was evident that both parental genomes did not display the same meiotic rhythm. Genomes organizing their own metaphase plate and the presence of two nucleoli observed in natural accessions (Mendes et al., 2006; Risso-Pascotto et al., 2006b; Boldrini et al., 2009a,b, 2010), and in interspecific artificial *Brachiaria* hybrids (Mendes-Bonato et al., 2006b; Adamowski et al., 2008) as well as in nonaploid accessions of *B. humidicola* (Boldrini et al., 2009a) also show that these parental genetic systems are not able to share the same cytoplasm immediately after hybridization and some accommodations are required for these genotypes to be successful.

Reduced fertility is commonly thought to constrain the demographic success of newly formed polyploids (Ramsey and Schemske, 2002). These authors point out that, broadly, three causes of sterility have been identified: i) meiotic abnormalities; ii) genetic causes that are independent of meiotic aberrations, and iii) incidental phenotypic effects of polyploidy, but meiotic aberrations probably represent the most general factor affecting polyploid fertility. Estimates of pollen viability in D53 and D71 revealed that only the former was reactive to propionic carmine staining (Figure 1o). A total of 10.3% of pollen grains in D53 were stainable and most likely, viable, but 100% of tetrads presented micronuclei (Figure 1j) or were fragmented into polyads. The occurrence of the first pollen mitosis in some pollen grains of this accession (Figure 1l,m,n) show that despite the elimination of one genome of nine univalents in micronuclei, the ability of the other genome ($2n = 36$) to progress in pollen development was not apparently compromised. If these pollen grains are truly fertile, the genome of nine univalents

acquired through hybridization would not be transmitted to the progeny in the case of sexual reproduction. Both accessions under analysis are apomictic, as most polyploid *Brachiaria* accessions are (Valle and Savidan, 1996; Valle and Pagliarini, 2009).

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