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Tetraploid races of *Paspalum notatum* show polysomic inheritance and preferential chromosome pairing around the apospory-controlling locus

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Abstract The objective of this work was to determine the type of inheritance (disomic/polysomic) in tetraploid ($2n$=$4x$=40) *Paspalum notatum* and investigate the transmission pattern of the chromosome region associated with apospory. An F_1 family segregating for the reproductive mode (aposporous vs non-aposporous) was generated by crossing a tetraploid sexual plant as female parent with an apomictic individual as pollen donor. Pollen mother cells from both parental plants were examined to ascertain chromosome-pairing behavior at meiosis. The high rate of quadrivalent chromosome associations indicated an autotetraploid origin of the species, although bivalent pairing and occasional univalents were detected. The observation of a lagging bivalent, a bridge of chromatin, or two aligned laggards in the aposporous parent suggested a chromosome inversion in this strain. Segregation ratios of AFLP markers and the proportion of linkages in repulsion versus coupling phase denoted tetrasomic inheritance, but markers displaying disomic ratios were also observed. Preferential chromosome pairing (disomic inheritance) in the chromosome segment related to apospory was detected. The possible relationship between a chromosome rearrangement and the inheritance of apospory is discussed.

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Introduction

Paspalum notatum Flügge (bahiagrass) is a perennial, rhizomatous forage grass native to South America. It is an important component of the natural grasslands of northeastern Argentina, southern Brazil, and Paraguay and has excellent potential for forage production (Burton 1948). *P. notatum* is considered an agamic complex where races with chromosome numbers $2n$=$2x$=20, $3x$=30, $4x$=40, and $5x$=50 have been found (Burton 1946; Gould 1966; Tischler and Burson 1995). The diploid form (*P. notatum* var. *saurae* Parodi) is sexual and meiotically stable with ten bivalents at meiosis, while polyploids reproduce by obligate aposporous apomixis (Forbes and Burton 1961). Apomixis in *P. notatum* is characterized by the complete degeneration of the products of meiosis, followed by the formation of embryo sacs from the nucellar cells (apospory), the development of embryos from cytologically unreduced egg cells (parthenogenesis), and the fertilization of the central nuclei to develop the endosperm (pseudogamy). Aposporous embryo sacs differentiate from meiotic embryo sacs by the lack of antipodal cells (Ortiz et al. 1997). Apomictic seeds lead to the generation of maternal offspring that are genetically identical to the mother plant.

Tetraploid races of *P. notatum* have been designated as autotetraploids based on their chromosome-pairing behavior at meiosis and their chromosome configurations in hybrids with the diploid form (Forbes and Burton 1961; Quarin et al. 1984). The determination of the type of inheritance (i.e., disomic or tetrasomic) is critical for elucidating the phylogeny and developing appropriate breeding strategies. Segregation ratios of molecular mark-

ers are thought to be an accurate method for assessing the type of ploidy, with polysomic ratios indicating autopolyploidy and disomic ratios indicating allopolyploidy (Qu and Hancock 1995). Likewise, the ratio between the numbers of loci linked in repulsion versus coupling phase has also been proposed (Sorrells 1992; Wu et al. 1992). Molecular markers were used to determine the polyploid type in *Saccharum spontaneum* (Da Silva et al. 1993), *Vaccinium darrowi* (Qu and Hancock 1995), and *Paspalum simplex* (Pupilli et al. 1997).

Genetic analysis of the inheritance of apospory in tetraploid *P. notatum* showed a strong distortion in the segregation ratio, probably due to a pleiotropic lethal effect with incomplete penetrance or a linked, partially lethal factor affecting the male gametes (Martínez et al. 2001). Moreover, suppression of recombination around the apospory locus was recently reported by Martínez et al. (2003). Genetic analyses of the transmission of apospory in *P. simplex* (Pupilli et al. 1997) and *Pennisetum* inter-specific hybrids (Ozias-Akins et al. 1998) indicated tetrasomic inheritance. However, in buffelgrass (*Pennisetum ciliaris*) apospory showed a disomic segregation ratio (Jessup et al. 2002).

The objective of this work was (1) to determine the type of inheritance present in tetraploid races of *Paspalum notatum* and (2) to investigate the segregation pattern of the chromosome segment carrying the control for apospory.

Materials and methods

Plant material

A tetraploid ($2n$=$4x$=40) F_1 population of 113 individuals, segregating for the reproductive mode, was generated by crossing the completely sexual plant Q4188 (Quarin et al. 2003) as pistillate parent with a natural apomictic individual of accession Q4117 as pollen donor, using the same procedure described in Martínez et al. (2001). The reproductive mode of each F_1 was determined by assaying two RAPD markers (UBC243-377 and UBC259-1157) completely linked to apospory in the species and then by examining the embryo sacs as described in Martínez et al. (2001, 2003).

Molecular markers

DNA extraction was performed by using the CTAB method according to Martínez et al. (2003). RAPD experiments were carried out as described previously (Martínez et al. 2003), using two primers from the University of British Columbia, UBC243 and UBC259. AFLP markers were generated according to Ortiz et al. (2001) and named with the prefix ex/mx (x=1 to n), designating the specific *Eco*RI and *Mse*I primers (from KeyGene, AFLP Protocol for Public Release, version 2.0, The Netherlands, 1994) used during the selective amplification and a final letter to differentiate fragments generated from the same primer combination.

Data analysis

As a result of using a tetraploid F_1 mapping population derived from non-inbred parents, different allelic configurations per locus were expected (Ritter et al. 1990). Data from each parental genotype were analyzed independently. Bands that segregated as single- or double-dose amplification fragments (SDAF and DDAF, respectively) were scored. A χ^2 test was used to determine the goodness of fit between the observed and the expected number of genotypes for each class of segregation ratio. A 1:1 (presence:absence) ratio is expected for markers present in single dose for both disomic or polysomic inheritance. Ratios of 3:1 or 5:1 are expected for double-dose alleles in cases of disomic or polysomic inheritance, respectively (Wu et al. 1992). Repulsion-phase markers were generated by recoding the presence and absence data set of each SDAF according to Al-Janabi et al. (1993). Linkages were detected by using Mapmaker 3.0 (Lander et al. 1987) at LOD=4.0 and a default linkage value of 0.39 [the maximum detectable recombination fraction (max$R$) for a population of 113 individuals in case of autopolyploidy (Wu et al. 1992)]. SDAF were treated as F_2-backcross data, and map units in centiMorgans were derived from the Kosambi (1944) mapping function. Repulsion-phase-linked markers were first investigated within each linkage group. Afterward, a two-point analysis was carried out with the complete data sets to detect other possible repulsion-phase linkages not included in the defined linkage groups (Qu and Hancock 2001). Ratios (linkages in repulsion vs coupling phase) of 1:1 or 0–0.25:1 are expected in cases of allo- or autopolyploidy, respectively (Wu et al. 1992). The occurrence of random or preferential chromosome pairing was estimated as described by Qu and Hancock (2001) for repulsion-phase-linked markers.

Results

Classification of F_1 plants for the mode of reproduction

Classification of F_1 progenies for the mode of reproduction by RAPD analysis showed that both markers (UBC243-377 and UBC259-1157) were consistently present in the apomictic parent Q4117 and in 15 F_1 plants and absent in the female progenitor Q4188 and in 98 F_1 plants in at least two independent experiments. Cytoembryological analysis confirmed the reproductive behavior of each progeny. The trait showed a strong deviation from the 1:1 sexual versus apomicts ratio ($\chi^2_{1:1}$=60.96, P< 0.01) expected for a single allele.

Table 1 Meiotic chromosome behavior in tetraploid ($2n$=$4x$=40) *Paspalum notatum* lines Q4188, the sexually reproducing female parent and Q4117, the pollen-donor apomictic parent. *PMC* Pollen mother cell

Strain	Number of PMCs analyzed	Mean chromosome associations per PMC at diakinesis and metaphase I				PMCs with univalents at diakinesis and metaphase I (%)	PMCs with one or two laggards at anaphase I[a] (%)	PMCs with micronuclei at telophase I[a] (%)
		I	II	III	IV			
Sexual Q4188	26	0.5	9.8	0.1	4.9	26.9	30.7	26.9
Apomictic Q4117	36	0.08	11.2	0.05	4.36	5.5	44.4	35.3

[a] Minimum of 20 cells were observed

Ploidy type and chromosome-pairing analysis

Both sexual and apomictic parental plants had 40 chromosomes that associated mainly forming bivalents and quadrivalents at diakinesis of metaphase I. On average, 43.6% (range, 20–80%) of the chromosomes formed quadrivalents in Q4188 and 49% (range, 10–80%) in Q4117. The average chromosome configuration per pollen mother cell (PMC) is shown in Table 1, (Fig. 1a–c). In Q4188, 26.9% of PMCs showed one or two univalents (Table 1, Fig. 1a), while only 5.5% of PMCs were observed in Q4117 (Table 1). Most anaphase I cells showed regular chromosome distribution (Fig. 1d), though lagging chromosomes were detected. In Q4188, 30.7% of anaphase cells had one or two lagging chromosomes, a proportion that was in agreement with the amount of meiocytes that showed univalents (26.9%) at diakinesis and metaphase I. In the apomictic plant, 44.4% of anaphase I cells showed lagging chromosomes. This proportion was unexpected since only few meiocytes (5.5%) had shown univalents. These laggards in Q4117 were: one bivalent lagging in the equatorial plate (Fig. 1e); a bridge (Fig. 1f); two single face-to-face chromosomes (Fig. 1g); or less frequently, two chromosomes joined by a bridge of chromatin. Interestingly, when two single chromosomes lagged at anaphase I, they were always located "face-to-face," indicating a delayed separation. In Q4188, the two lagging univalents were usually located randomly in the cytoplasm (Fig. 1h). At telophase I, both Q4188 and Q4117 showed mostly two groups of chromosomes clumped in the poles (Fig. 1i), though one or two micronuclei were observed in 26.9% and 35.3% of the meiocytes, respectively (Table 1). In some meiocytes of Q4117, the micronucleus observed was extremely small, suggesting that it might be originated from a chromosome fragment (Fig. 1j). The presence of bridges of chromatin with accompanying fragments is an indirect evidence for existence of inversions (Brown 1972).

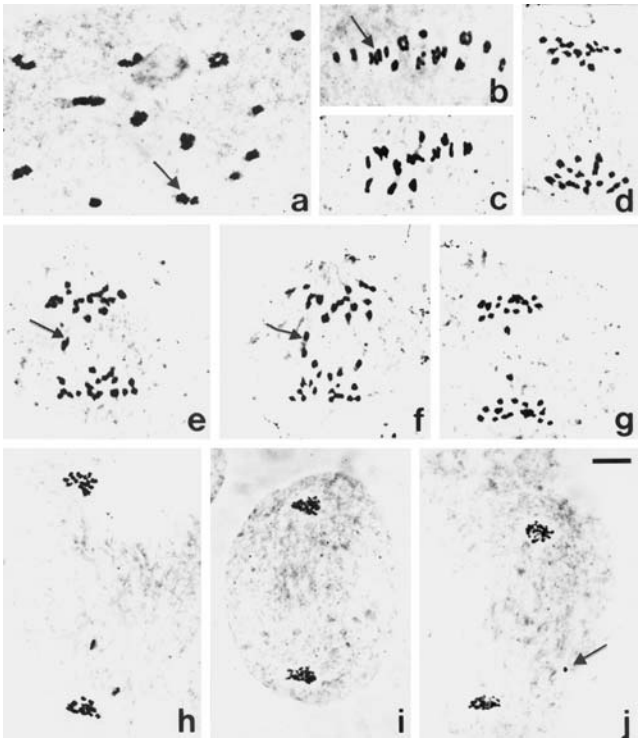

Fig. 1 Meiosis in sexual (Q4188) and apomictic (Q4117) parental lines of *Paspalum notatum*: (a) diakinesis in sexual Q4188 with 1 I + 4 II + 1 III (arrow) + 7 IV. (b–g) apomictic Q4117. (b) metaphase I with 12 II + 4 IV, arrow points to three bivalents close together. (c) metaphase I with 4 II + 8 IV. (d) late anaphase I. (e) anaphase I with a lagging bivalent (arrow). (f) anaphase I with a bridge (arrow). (g) anaphase I with two face-to-face lagging chromosomes. (h) telophase I in sexual Q4188 with two lagging chromosomes. (i–j) apomictic Q4117. (i) regular telophase I. (j) telophase one with a small lagging chromatin body (arrow). Bar = 10 microns

Genetic analysis of AFLP markers

Twenty-four primer combinations were used to generate AFLP markers. A total of 219 and 176 amplification bands, segregating from the female and male parent, respectively, were scored (Table 2). The presence of at least ten AFLP bands segregating from Q4117 confirmed the hybrid origin of 113 F_1 plants. The number of markers scored in each class of segregation ratio is shown in Table 2. Nine DDAFs from both Q4188 and Q4117 segregated with values that fit exclusively to a 5:1 ratio. Other groups of 26 DDAFs segregated in a 5:1 ratio, but were not statistically distinguishable from a 3:1 ratio (Table 2). All these markers indicated polysomic inheritance for both sexual and apomictic strains. Nevertheless, 17 DDAFs from Q4188 and 21 from Q4117 segregated exclusively in a 3:1 ratio (Table 2).

To assess the relative proportion of markers in each linkage phase (coupling and repulsion) segregation data from SDAF from each parent were combined with its

Table 2 Number of AFLP markers scored in each class of segregation ratio from both sexual (Q4188) and apomictic (Q4117) tetraploid *P. notatum*. *SDAF* Single-dose amplification fragment, *DDAF* double-dose amplification fragment

Genotypes	Segregation ratio					Total
	SDAF	DDAF			Distorted (%)	
	1:1 (%)	3:1 (%)	3:1 or 5:1 (%)	5:1 (%)		
Q4188	167 (76.2)	17 (7.8)	10 (4.6)	9 (4.1)	16 (7.3)	219
Q4117	106 (60.2)	21 (11.9)	16 (9.1)	9 (5.1)	24 (13.7)	176

[a] $P<0.01$

Table 3 Repulsion-phase-linked markers in tetraploid sexual (Q4188) and apomictic (Q4117) *P. notatum.* Only markers segregating in A 1:1 ratio (P>0.01) from each parental plant were used. Repulsion-phase linkages were detected as described in "Material and methods" by using the mapping program MAPMAKER 3.0 (Lander et al. 1987) at a LOD score of 4.0 and a maximum recombination value of 0.39

Pair-wise linked markers	Theta[a]	CentiMorgans[b]	r_2[c]	LOD
Q4188 (sexual)				
e42m37g/e39m42i[d]	0.29	32.5	–0.024	4.61
e32m36e/e39m42i[d]	0.29	32.5	–0.024	4.61
e32m36b/e39m42i[d]	0.26	28.7	–0.138	5.90
e34m36n/e33m42e	0.28	31.2	–0.063	5.02
Q4117 (apomictic)				
e32m33a/e34m43 h	0.27	30.0	–0.099	5.22
e32m33c/e35m33c	0.29	32.9	–0.012	4.17
e41m41m/e41m41n	0.28	31.2	–0.063	5.02

[a] Recombination fraction
[b] Kosambi's mapping function (R)
[c] r_2 Actual genetic distance of markers linked in repulsion phase = $(h-1)\times(R-R_i)/100$, where h = number of homologs in a group (Qu and Hancok 2001). R_i for an autotetraploid is 33.3 (Wu et al. 1992)
[d] Pairs of markers belonging to the same linkage group

inverted form and analyzed by using Mapmaker 3.0. The maternal file contained 334 loci and the paternal file 212. Linkage maps of each parental genotype were constructed (not shown) at a minimum LOD score of 4.0 and a maximum recombination value of 0.39. The maternal map contained 12 linkage groups of three or more markers, while the paternal map had 15 groups (not shown). On average, adjacent markers showed minimum and maximum recombination values of 2.7 cM and 33.8 cM, respectively. Two linkage groups containing one and three pairs of markers linked in repulsion phase were detected in the maternal map, while three linkage groups carrying single pairs of repulsion-phase-linked markers were identified in the paternal map (Table 3). Two-point linkage analysis with the complete data sets showed no other pair of markers linked in repulsion phase. The actual proportions between linkages in repulsion versus coupling phase were 0.008:1 (4/522) and 0.048:1 (3/212) for the maternal and paternal maps, respectively. These are distinctive ratios for polysomic inheritance (Qu and Hancock 2001). Moreover, since recombination values for most of the repulsion-phase linkages were not significantly different from the R_i (33.3 cM), a random association of homologs is assumed for the species (Table 3).

Linkage analysis of the 16 markers segregating from the maternal genotype with distorted segregation ratios showed four and five markers clustered in two different linkage groups, three scattered on other three groups, and the remaining four markers stayed unlinked.

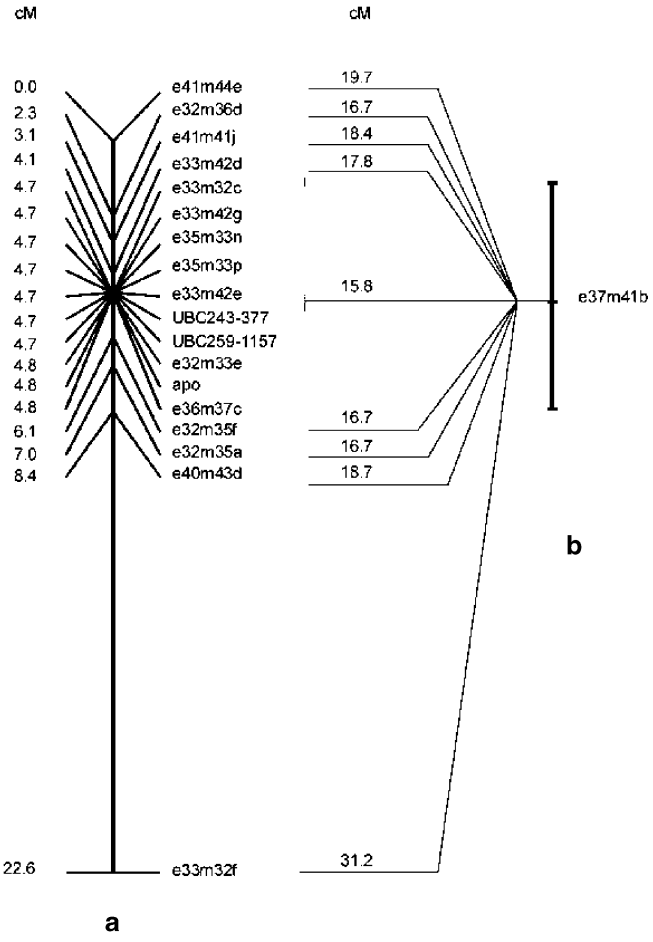

Fig. 2 Paternal linkage group carrying the locus responsible for apospory (apo). AFLP markers names and map distance in cM (Kosambi) are shown to the right and left, respectively. a and b represents homologous chromosomes with markers linked in repulsion-phase. Linkage analysis was carried out by using the mapping program MAPMAKER 3.0 at LOD score = 4.0 and a maximum recombination value of 0.39

Genetic analysis of markers associated with apospory

Considering the strong distortion in the segregation ratios observed for apospory and 24 AFLP markers segregating from the apomictic parent ($7.57<\chi^2_{1:1}<87.72$), an independent genetic study was carried out for these loci. Data from RAPDs markers (UBC243-377 and UBC259-1157) used for determining the reproductive mode of each progeny were also included. Linkage analysis showed that the apospory locus and 17 markers mapped together to a single coupling-phase linkage group (apospory group) (Fig. 2). The apospory group covered 22.6 cM where nine markers mapped completely linked to apospory, and eight other markers were located at both sides of the apospory locus. This group of markers defined an apospory-linkage block in which 16 adjacent markers mapped over 8.4 cM.

Table 4 Repulsion-phase-linked markers mapping to the linkage group carrying the locus responsible for apospory in tetraploid *P. notatum* strain Q4117. Segregation data from apospory and AFLP markers showing distorted segregation ratios ($P<0.01$) were used (Table 2). Repulsion-phase linkages were detected as described in "Material and Methods" by using the mapping program MAPMAKER 3.0 (Lander et al. 1987) at LOD score of 4.0 and a maximum recombination value of 0.39

Pair-wise linked markers	Theta[a]	cM[b]	r_2[c]	LOD
apospory/e37m41b[d]	0.15	15.8	−0.525	12.78
e32m35a/e37m41b	0.16	16.7	−0.498	12.27
e32m36d/e37m41b	0.16	16.7	−0.498	12.27
e40m43d/e37m41b	0.18	18.7	−0.438	10.89
e41m41j/e37m41b	0.18	18.4	−0.447	10.69
e41m44e/e37m41b	0.19	19.7	−0.408	10.24
e32m35f/e37m41b	0.16	16.7	−0.498	12.27
e33m42d/e37m41b	0.17	17.8	−0.465	11.35
e33m32f/e37m41b	0.28	31.2	−0.063	5.02

[a] Recombination fraction
[b] Kosambi's mapping function (R)
[c] r_2 Actual genetic distance of markers linked in repulsion phase = $(h-1)\times(R-R_i)/100$, where h = number of homologs in a group (Qu and Hancok 2001). R_i for an autotetraploid is 33.3 (Wu et al. 1992)
[d] The same value correspond to pairs UBC243-377/e37m41b; UBC259-1157/e37m41b; e32m33e/e37m41b; e36m37c/e37m41b; e33m32c/e37m41b; e33m42e/e37m41b; e33m42g/e37m41b; e35m33n/e37m41b; and e35m33p/e37m41b

The complete cosegregation between nine markers and the apospory locus was in agreement with the suppression of the recombination in this chromosome segment reported by Martínez et al. (2003). It seems to be unlikely that we would have saturated a small chromosome region with a mapping population of 113 individuals and a random strategy for generating the markers. The remaining markers unrelated to apospory mapped scattered in the genome, two of them mapping to different linkages groups, and the rest staying unlinked. Genetic analysis for detecting linkages in repulsion phase within the group showed that the apospory block was linked to a single marker (e37m41b) (Fig. 2). Recombination values (R) between all loci from the apospory block and marker e37m41b ranged from 15.8 to 19.7, all less than the expected R_i (33.3). Consequently, negative values for r_2 were produced, indicating preferential chromosome pairing or disomic inheritance in this chromosome region (see Table 4).

Discussion

Cytogenetic analysis of both sexual and apomictic tetraploid *Paspalum notatum* strongly indicated an autotetraploid origin of the species as it was proposed by Forbes and Burton (1961) and Quarin et al. (1984). However, since the proportion of chromosomes forming quadrivalents varied greatly and frequently bivalents were observed, both disomic and tetrasomic segregation ratios could be expected from these strains.

Analysis of DDAFs showed several loci segregating with a polysomic ratio (5:1). This result indicated tetrasomic inheritance in the species, since it can only be explained considering a random assortment of the four homologs. However, a considerable number of DDAFs with segregation values that fit exclusively to a 3:1 ratio was detected. A possible explanation would be that these markers were located in genomic regions where preferential chromosome pairing or disomic inheritance occurs. As DDAFs cannot be mapped by conventional methodology, we were unable to assign these markers to any of the defined linkage groups. The alternative hypothesis of segmental allopoliploidy is worst sustainable, according to the cytological observations. Moreover, the ratios between the numbers of markers linked in repulsion versus coupling phase confirmed the tetrasomic inheritance and a random chromosome pairing at meiosis.

Cytological analysis of strain Q4117 suggested the presence of an inverted segment concerning one chromosome. This chromosome rearrangement might be a paracentric inversion, located close to the centromere, since the homologous chromosomes of one bivalent frequently remained tightened beyond metaphase I or had delayed separation. As it is well known that inversions can cause gamete lethality and suppression in crossing over within the inverted chromosome segment (Brown 1972), the presence of an inversion in Q4117 could be responsible for both the distorted segregation ratio observed in the transference of apospory (and a group of markers linked to it) and the suppression of recombination observed around the apospory-controlling locus. No evidence for inversions was detected in the sexual genotype Q4188. Future analyses with fluorescence in situ hybridization, like the study recently reported by Goel et al. (2003), may help to determine whether the apospory linkage block is placed on the lagging bivalent chromosome pair observed at early meiotic anaphase I and explain the lack of recombination around apospory. Preferential chromosome pairing (disomic inheritance) of the chromosome segment carrying the control for apospory was detected. This finding indicates that the apospory block has homology with a segment placed in only one of the other three chromosomes of the set. This characteristic may account for the detection of several markers flanking the apospory locus with low recombination ratios in an autotetraploid species. Similar results were reported in buffelgrass (Gustine et al. 1997) and *Brachiaria* (Pessino et al. 1998) and support the idea that recombinants near the apospory locus may only be recovered from some crosses (Jessup et al. 2002).

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