Plant Syst Evol
DOI 10.1007/s00606-016-1345-z

ORIGINAL ARTICLE

Hybridity and autopolyploidy increase the expressivity of apospory in diploid *Paspalum rufum*

Luciana Delgado[1] · María E. Sartor[2] · Francisco Espinoza[2] · Mariano Soliman[1] · Florencia Galdeano[2] · Juan P. A. Ortiz[1]

Received: 3 May 2016 / Accepted: 4 August 2016

Abstract *Paspalum rufum* Nees is a perennial grass that forms a multiploid complex. Natural populations are composed mainly of diploid ($2n = 2x = 20$) and tetraploid ($2n = 4x = 40$) cytotypes. The diploid form is sexual and highly self-sterile, while the tetraploid is pseudogamous aposporous apomict and self-fertile. Diploids can also develop aposporous sacs, and some of them complete apomixis. The objective of this work was to analyse apospory expressivity (a gametophytic apomixis component) in diploid hybrids and colchicine-induced autotetraploids of the species. One F_1 family was created by crossing two diploid individuals (R6#45 × R5#49) carrying aposporous sacs in 5.8 and 13 % of their ovules, respectively. Moreover, two synthetic autotetraploids were obtained by colchicine treatment of mature seeds from R6#45. The hybrid origin of the F_1s was confirmed by segregation analyses of a morphological trait and molecular markers, and the ploidy level of experimental plants was determined by flow cytometry. Apospory expressivity was estimated by embryo sacs observation at anthesis. Out of the 39 hybrids analysed, 38 showed aposporous embryo sacs. Expressivity of the trait ranged from 0 to 36 %, and some individuals differed significantly for both progenitors' values. Both doubled-diploid plants showed 25 and 32 % of apospory expressivity, which was significantly higher than that observed in R6#45. Results presented in this work revealed a high variability in apospory expressivity of diploid hybrids and suggested that more than one allele are controlling the trait. Moreover, the new induced doubled-diploid plants showed that apospory expressivity is highly ploidy dependent.

Keywords Apospory expressivity · Diploid · *Paspalum rufum* · Polyploid

Handling editor: Martin Lysak.

Electronic supplementary material The online version of this article (doi:10.1007/s00606-016-1345-z) contains supplementary material, which is available to authorized users.

✉ Luciana Delgado
luciana.delgado@conicet.gov.ar

[1] Instituto de Investigaciones en Ciencias Agrarias de Rosario (IICAR) CONICET-UNR, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Parque Villarino s/n CC 14, S2125ZAA Zavalla, Santa Fe, Argentina

[2] Instituto de Botánica del Nordeste (IBONE) CONICET–UNNE, Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste, Sgto. Cabral 2131, 3400 Corrientes, Argentina

Introduction

Paspalum, with near 350 species, is one of the largest genus of the *Poacea* family (Zuloaga and Morrone 2005). Most of them have extremely versatile genetic systems that combine variations in chromosome number, meiotic chromosome behaviour, and reproductive modes (Ortiz et al. 2013). Several species form multiploid complexes where diploids are sexually self-sterile and polyploids are self-fertile pseudogamous apomicts (Quarin 1992). Apomixis, i.e. asexual reproduction through seeds, is widespread in the genus (Quarin 1992). This type of reproduction generates offspring which are normally exact genetic copies of the mother plant (Nogler 1984). Thus, apomixis offers a unique opportunity to fix superior genotypes and hybrids. Seeds of any superior obligate apomict could be multiplied by unlimited number of open-pollinated generations without loss of vigour or genotype change (Hanna and Bashaw 1987). *Paspalum* spp. has gametophytic-type apomixis,

Published online: 22 August 2016

mainly aposporic (Quarin 1992). Unreduced embryo sacs differentiate from nucellar cells—called aposporous initials (AIs)—which, after a series of mitotic divisions, develop into mature embryo sacs containing a non-reduced ($2n$) egg cell, one or two synergids, and a binucleated central cell. Antipodals are always absent in these so-called *Paspalum*-type aposporous embryo sacs (AES) (Ortiz et al. 2013). Instead, sexuality is characterized by meiotic embryo sacs of the *Polygonum* type, typically composed of the egg apparatus (the egg cell and two synergids), a large two-nucleated central cell, and a mass of proliferated antipodals at the chalazal end (Quarin 1992). Although gametophytic apomixis is strongly associated with polyploidy, several diploid species of *Paspalum* generate a low proportion of ovules containing AES together with the typical meiotic sac or even single aposporous sacs (Norrmann et al. 1989; Quarin 1986; Quarin et al. 2001; Quarin and Norrmann 1987).

Paspalum rufum Nees is a robust erect perennial native grass of Paraguay, southern Brazil, Uruguay, and north-eastern Argentina. It is usually found in marshes, wet savannahs, or low open grounds forming large clumps of characteristic light green colour (Quarin et al. 1998). Natural populations are mainly composed by diploids ($2n = 2x = 20$) that reproduce sexually and tetraploids ($2n = 4x = 40$) that reproduce by facultative aposporous apomixis (Sartor et al. 2011). Nevertheless, previous works have confirmed the presence of AES in diploids and have proposed that they play a role in new apomictic polyploids formation (Quarin and Norrmann 1987; Siena et al. 2008). A recent survey in natural diploid populations showed that apospory is frequently expressed (ranging from 1.8 to 13 %), and some individuals are able to produce up to 15 % of their progenies by apomixis in intra-specific and inter-ploid crosses (Delgado et al. 2014). These findings indicate that genetic determinant/s of the apomixis components is/are present in diploids, but its/their expressivity remains inhibited. Support for this idea also comes from the results obtained after diploid chromosome doubling of *P. notatum* var. *saurae* that allowed the recovery of facultative apomictic tetraploids (Quarin et al. 2001). Thus, polyploidization might act either on the apomixis controlling locus, through some transcription factors, or via a secondary locus that requires a higher allele dosage to affect the expression of the main locus (Quarin et al. 2001).

Often, both apomictic and sexual pathways coexist in the same plant at different rates (Spillane et al. 2001). In several species of *Paspalum,* most polyploids are facultative apomictic, displaying low proportions of meiotic embryo sacs (MES). However, polyploid accessions producing as high as 63.8 % of MES have also been reported (Espinoza et al. 2006; Martínez et al. 2001; Quarin et al. 1984, 2001; Sartor et al. 2011). Variation in apomixis expressivity represents a serious problem for breeding programs intending to generate apomictic hybrid cultivars. Thus, the analysis of the inheritance and expressivity of this trait will help to understand the variations observed and will contribute to the development of obligated apomictic cultivars. The objectives of this work were to analyse the expressivity of apospory in diploid hybrids of *P. rufum* derived from two natural genotypes carrying AES, as well as in two synthetic autotetraploids obtained by colchicine treatment of seeds from a diploid plant of the species.

Materials and methods

Plant material and generation of hybrids

Two diploid ($2n = 2x = 20$) individuals (R6#45 and R5#49) of *P. rufum* collected from natural populations from north-eastern Argentina were used. The reproduction mode of both genotypes had previously been characterized by Delgado et al. (2014). Briefly, R6#45 is a self-sterile individual that produces all its seeds by sexuality, irrespective of the pollen source (Delgado et al. 2014). It showed 5.8 % of its ovules containing one MES along with one or two AES (Delgado et al. 2014). This plant has light yellow anthers. R5#49 is a self-sterile plant with the ability to reproduce by different reproductive strategies. It forms seeds by sexuality in crosses with diploids, but generates B_{II} and B_{III} hybrids, as well as S_{III} and maternal (apomictic) seeds after intra-specific inter-ploidy crosses and inter-specific inter-ploidy self-pollination induction (Delgado et al. 2014). This individual showed 13 % of its ovules bearing MES along with one or more AES or single AES (Delgado et al. 2014). R5#49 has dark yellow anthers. Both genotypes are expected to be highly heterozygous due to their self-sterility (Delgado et al. 2014). Crosses were carried out without emasculation of the plants, using R6#45 as pistillate parent and R5#49 as pollen donor. Filled seeds were germinated in sterilized soil, and seedlings were planted in small pots in a greenhouse. Afterwards, the plants were transferred to a field nursery and grown under natural conditions at the Agronomy College of the National University of Rosario, Argentina.

Molecular markers assays

Seventeen RAPD primers from the UBC series (UBC301–310, UBC322, UBC329, UBC347, UBC349, UBC359, UBC361, and UBC368) were screened in order to detect polymorphic markers between genotypes R6#45 and R5#49 for hybrid identification. Amplification reactions were carried out according to Siena et al. (2008).

After screening, six primers were selected for analysing the entire F_1 population. Each plant was scored for the presence of pollen donor-specific bands as well as for segregating exclusive female parent bands. Individual plants were considered diploid hybrids if they showed, at least, two bands from R5#49.

Colchicine treatments and ploidy level determination

Seeds from R6#45 open pollination and controlled crosses (with the diploid individual R2#8) were used for chromosome duplication. Spikelets from dissected seeds were incubated for 24 h at 27 °C to initiate germination in petri dishes containing wet filter papers imbibed with sterile distilled water. Afterwards, caryopses were transferred to petri dishes containing filters moistened with fresh colchicine solution (0.1 % w/v) plus 2 % dimethyl sulphoxide (DMSO) and incubated for 6, 12, 24, and 30 h. At least 50 seeds were included in each treatment. The caryopses were then washed with sterile distilled water and transferred to petri dishes with filters imbibed in distilled water to allow germination. Each treatment included controls replacing colchicine solution with sterile distilled water. Seedlings were individually transferred to pots with sterilized soil and kept in the greenhouse until analysis. The ploidy level of the experimental plants was determined by flow cytometry according to Sartor et al. (2011) using a diploid plant of *P. rufum*, accession Q3774, as internal standard. Briefly, fresh leaf tissue (0.5 cm^2) was placed in small petri dishes with a similar amount of leaf tissue from the internal standard (Norrmann et al. 1989). The ploidy level was estimated by comparing the DNA peaks of the sample with the standard. Measurements were first taken for young leaves of each seedling and then for each leaf arising from the new tillers. If one of them showed leaves with ploidy increment, it was separated from the rest.

Mode of reproduction and expressivity of apospory

Inflorescences at anthesis were fixed in FAA (70 % ethanol, glacial acetic acid, formaldehyde, in a ratio 90:5:5) and transferred to ethanol 70 % for 24 h. Dissected pistils were clarified following the protocol described by Young et al. (1979). Ovules were observed with a light transmission Leica DIASTAR microscope equipped with a differential interference contrast (DIC) system and a digital camera (Leica Microsystems, Wetzlar, Germany). Embryo sacs showing the egg apparatus, two polar nuclei, and a group of antipodal cells were classified as meiotic of the *Polygonum*-like type. Embryo sacs showing the egg apparatus, two polar nuclei, but lacking antipodal cells were regarded as AES of the *Paspalum* type. Embryo sacs showing low development were classified as aborted (Ab) (Siena et al. 2008). At least 29 ovules per plant were scored for the presence of single MES, single or multiple AES, or one MES plus one or more AES (MES + AES). The expressivity of apospory was estimated as the proportion of ovules carrying at least one AES over the total scored ovaries.

Statistical analysis

A Chi-square test was used to determine the goodness of fit between observed and expected segregation values for the morphological trait and RAPD molecular markers. Ratios that differed from the expected value at $p < 0.05$ were classified as distorted. Confidence intervals (CIs) around observed proportions of AES of each plant were calculated following the method described by Newcombe (1998), derived from a procedure outlined by Wilson (1927) without correction for continuity using the online resource http://vassarstats.net/. The Fisher exact test (Upton 1992) was used for determining differences in the proportions of AES between experimental plants. Calculations were performed by using the MeasuringU online source (http://www.measuringu.com/ab-calc.php).

Results

Development of the F_1 population and hybrid identification

A total of 41 seedlings were recovered after the seeds derived from the experimental cross between R6#45 and R5#49 had been germinated. Plants were established in the field and allowed to open pollinate (Fig. 1a). Hybrids were morphologically very similar, but the anthers' colour segregated in the population (Fig. 1b–d). A total of 17 light yellow plant anthers (similar to the R6#45) and 22 dark yellow anther individuals (resembling R5#49) were observed. Accordingly, the dark yellow trait segregated as a single heterozygous dominant locus ($\chi^2_{1:1;\ 1.d.f.\ =}$ 0.621, $p = 0.423$) and suggested that dark yellow anther F_1 plants were of hybrid origin. Screening of RAPD primers showed that 6 out of the 17 assayed revealed clear polymorphism between genotypes (Online Resource 1). Markers showing polymorphisms (UBC302, UBC304, UBC306, UBC308, UBC310, and UBC322) were then used to analyse the entire F_1 population. Amplification with this set of primers allowed the generation of 12 segregating markers (eight from the pollen donor, two from the pistillate parent, and two from both parental plants). All markers but one segregated according to the expected values for heterozygous single alleles, indicating that they could be used for hybrid

Fig. 1 F_1 population derived from a cross between R6#45 and R5#49 genotypes of *Paspalum rufum*. **a** F_1 individuals in the field nursery, **b** spike of a panicle from inflorescences of R6#45 at *right* and R5#49 at *left*, **c**, **d** details of anthers from R6#45 and R5#49, respectively

identification (Table 1). Experimental plants were scored for the presence of at least two markers segregating from the pollen donor. Following these criteria, all individuals were classified as hybrids as all of them presented at least two paternal markers and no progeny plant derived from selfing was detected. Additionally, ploidy level determination by flow cytometry confirmed that all hybrids were diploids (Online Resource 2). This examination was useful in order to avoid the inclusion, in the analysis, of any hybrid derived from unreduced female gametes (i.e. plants derived from the fertilization of the unreduced embryo sac of R6#45).

Mode of reproduction and expressivity of apospory

The mode of reproduction of the 39 surviving diploid hybrids was estimated by cytoembryological observation of

Table 1 Anther's colour and RAPD markers segregation analysis in the F_1 population of diploid *Paspalum rufum* derived from an experimental cross between genotypes R6#45 and R5#49

Locus	Number[a]	Maternal phenotype/band	Paternal phenotype/band	χ^2 (p)
Anther's colour	39	17	22	0.641 (0.423)
302 a ♂	39	21	18	0.231 (0.631)
304 ♂	39	20	19	0.026 (0.872)
306 a ♂	40	37	3	0.225 (0.635)
306 b ♀	41	26	15	2.951 (0.086)
306 c ♂	40	19	21	0.100 (0.752)
308♀	39	22	17	0.640 (0.420)
308♂	39	13	26	4.330 (0.040)
310a ♂	40	17	23	0.900 (0.343)
310 b ♂♀	38	27	11	0.316 (0.574)
310 c ♂♀	38	27	11	0.316 (0.574)
322 a ♂	41	0	41	0.000 (1.000)
322 b ♂	41	19	22	0.220 (0.640)

[a] Number of total individuals analysed; ♀ and ♂ indicate markers segregating from R6#45 and R5#49 genotypes, respectively

ovules at anthesis. Cleared ovules were analysed by the presence of MES, MES plus AES, or single AES. A minimum of 29 ovaries per plant were scored, but in those individuals where the proportion of AES was low (less than 0.05), the number of ovules examined was increased up to 112 (Table 2). Observations revealed that 38 (97 %) hybrids carried AES and only one (3 %) presented MES exclusively (Fig. 2a). In general, individuals with the capacity for apospory showed ovaries containing both a well-developed *Polygonum*-like embryo sac (MES) and one or more AES (Fig. 2b). In all cases, the MES was placed at the centre of the ovule with the egg apparatus well in front of the micropylar end. However, eight individuals showed a low proportion of their ovules bearing exclusively one or two AES, which were localized in the middle lower part of the ovule (Fig. 2c). The hybrid plant that showed MES exclusively was analysed in detail and over 100 ovules were examined. Apospory was detected in none of them, indicating a very low level or absence of apospory expression (Table 2). Expressivity of the trait had a wide range of variation (Table 2; Fig. 3). Values were almost continuous from 1 to 15 %, but gaps between 15–18 and 18–22 % were observed. Most hybrids showed proportions of ovules with aposporous sacs falling within the range of both parental genotypes. However, extreme values were detected in two plants (#15 and #39) that showed proportions of apospory significantly higher (32.76 and 35.85 %, respectively) than those observed in both progenitors. On the other hand, individuals #5 and #9 showed minimal expressivity of the trait (1.05 and 1.22 %, respectively), although proportions were non-statistically different from genotype R6#45 (Fig. 3). This kind of transgressive segregation suggested a high degree of heterozygosity at the locus/loci responsible for apospory. Moreover, according to the observed ratio for the presence/absence (apospory vs. non-apospory) of the trait ($\chi^2_{3:1;\ 1\text{d.f.}} = 10.47$; $p < 0.05$ and $\chi^2_{4:0;\ 1\text{d.f.}} = 0.026$, $p > 0.05$), apospory was not segregating in the population and thus must have been controlled by a multiallelic locus or by more than one locus.

Expressivity of apospory in colchicine-induced autotetraploids

In order to analyse the influence of the polyploidy in the expressivity of apospory in *P. rufum*, two duplicated individuals, named LD1 and LD3, were obtained by treating the mature caryopses of the diploid plant R6#45 with 0.1 % w/v of colchicine solution for 6 h (Table 3). Both plants were transferred to soil and grown under natural conditions (Fig. 4a). Non-surviving seedlings were recovered in the other treatment tested (not shown). The ploidy level of both plants was confirmed by flow cytometric analysis of young leaves. Histograms of both plants showed a peak corresponding to duplicated DNA (4Cx) content with respect to the internal control (2Cx) (Fig. 4b–e). Cytoembryological observations of 72 ovules from LD1 at anthesis showed that 23 of them (32 %) produced ovaries containing MES plus one or more AES. Likewise, from the 61 ovules analysed from genotype LD3, 11 (18 %) showed ovaries with one MES plus one or more AES, and 4 (6.55 %) displayed AES exclusively. Adding up these values gave an overall capacity for apospory of 24.6 % (Table 4). Confidence intervals for the proportions of ovules carrying aposporous sacs of both duplicated plants showed significant differences compared to the original R6#45 individual (Fig. 4f).

Discussion

Gametophytic apomixis is strongly associated with hybridization and polyploidy (Savidan 2000). However, the relative contribution of these processes to the expression of the trait is still not clear. The "hybridization theory" indicates that the combination of two genomes from species with different reproductive characters may contribute to the induction of apomixis (Carman 1997). Examples supporting this concept can be found in the *Boechera* complex (Aliyu et al. 2010) as well as in common dallisgrass *Paspalum dilatatum* (Burson 1991; Speranza 2009). However, several cytogenetic and molecular analyses in most apomictic species of *Paspalum* suggested that they have an autopolyploid rather than an allopolyploid origin (Bennett and Bashaw 1966; Hojsgaard et al. 2008; Norrmann et al. 1989; Pupilli et al. 1997; Stein et al. 2004). Moreover, apospory has been observed in several species of the genus at diploid level, and in the case of *P. rufum,* even the complete apomictic pathway was detected (Delgado et al. 2014; Norrmann et al. 1989; Quarin 1986; Quarin et al. 2001; Quarin and Norrmann 1987; Siena et al. 2008). Evaluation of control and regulation of apomixis components in diploid systems can help to understand the organization and evolution of the agamic complexes as well as to transfer the trait to diploid crops.

In the present work, we analysed the transmission and expressivity of apospory in diploid hybrids of *P. rufum* that were derived from two individuals with the capacity to form aposporous embryo sacs as well as in two synthetic autopolyploids obtained by colchicine treatment of a diploid. According to the self-incompatibility system present in diploids, both parental plants were assumed to be highly heterozygous. Consequently, a segregating F_1 family was obtained in which the anther's colour as well as most molecular markers segregated with values that fit with the expected model for single heterozygous loci.

Table 2 Cytoembryological analysis of diploid hybrids of *Paspalum rufum* derived from an experimental cross between genotypes R6#45 and R5#49

Individual	Number of ovule scored						%AES[a]
	Total	AbES	MES	AES	MES + AES	NC	
#12	112	10	96	0	0	6	0.00
#5	94	31	60	0	1	2	1.06
#9	82	9	70	0	1	2	1.22
#40	39	3	35	0	1	0	2.56
#30	115	3	100	0	3	9	2.61
#34	38	2	34	0	1	1	2.63
#21	33	6	25	0	1	0	3.03
#18	32	3	27	0	1	1	3.13
#17	31	1	29	0	1	0	3.23
#1	57	1	52	0	2	2	3.51
#28	87	4	54	1	3	25	4.60
#23	43	12	29	0	2	0	4.65
#36	96	1	75	0	5	15	5.21
#41	38	1	32	0	2	3	5.26
#37	38	0	36	0	2	0	5.26
#8	36	2	30	0	2	2	5.56
#19	35	5	27	0	2	1	5.71
#43	65	4	54	0	4	3	6.15
#22	65	1	59	0	4	1	6.15
#7	45	9	32	0	3	1	6.67
#35	29	5	12	0	2	10	6.90
#6	98	2	87	0	7	2	7.14
#33	38	3	32	1	2	0	7.89
#16	48	5	37	0	4	2	8.33
#2	57	1	50	0	5	1	8.77
#29	44	3	36	0	4	1	9.09
#38	42	4	34	0	4	0	9.52
#27	31	13	15	2	1	0	9.68
#20	35	4	26	4	0	1	11.43
#13	33	1	25	1	3	3	12.12
#4	39	0	34	0	5	0	12.82
#25	37	3	28	0	5	1	13.51
#42	43	2	33	0	6	2	13.95
#10	43	3	34	1	5	0	13.95
#24	40	14	20	5	1	0	15.00
#3	57	10	35	7	3	2	17.54
#31	36	2	26	0	8	0	22.22
#15	58	2	33	2	17	4	32.76
#39	53	5	27	2	17	2	35.85

AbES ovaries with aborted embryo sacs, *MES* ovaries showing meiotic embryo sacs, *AES* ovaries showing aposporous embryo sacs, *NC* non-classified

[a] Calculated as percentage of ovaries carrying AES over total ovaries scored

Segregation analysis with molecular markers showed that all plants were of hybrid origin. Moreover, determination of the DNA content of hybrids by flow cytometry confirmed that all hybrids were diploids. These outcomes confirmed the heterozygosity of both parental plants and indicated that in crosses between diploids, all progenies were derived by sexuality, i.e. from the double fertilization of MES by reduced pollen nuclei. Thus, although both parental plants had the capacity for apospory, neither B_{III} hybrids nor apomictic progenies were recovered after the

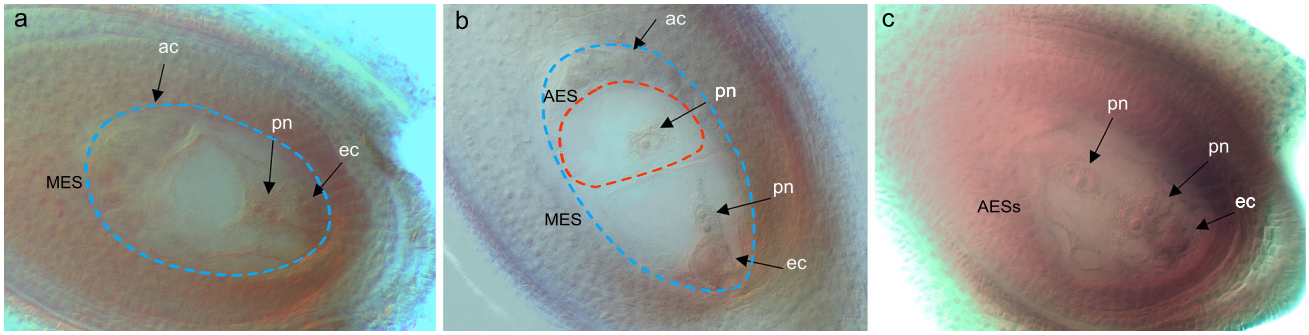

Fig. 2 Cytoembryological analysis of diploid hybrids of *Paspalum rufum*. **a** Ovary carrying a meiotic embryo sac, **b** ovary containing one meiotic embryo sac towards the micropylar pole and one aposporous embryo sac at the chalazal end, **c** ovary enclosing two aposporous embryo sacs. *pn* polar nuclei, *ac* antipodals cells, *ec* egg cell, *MES* meiotic embryo sac, *AES* aposporous embryo sac

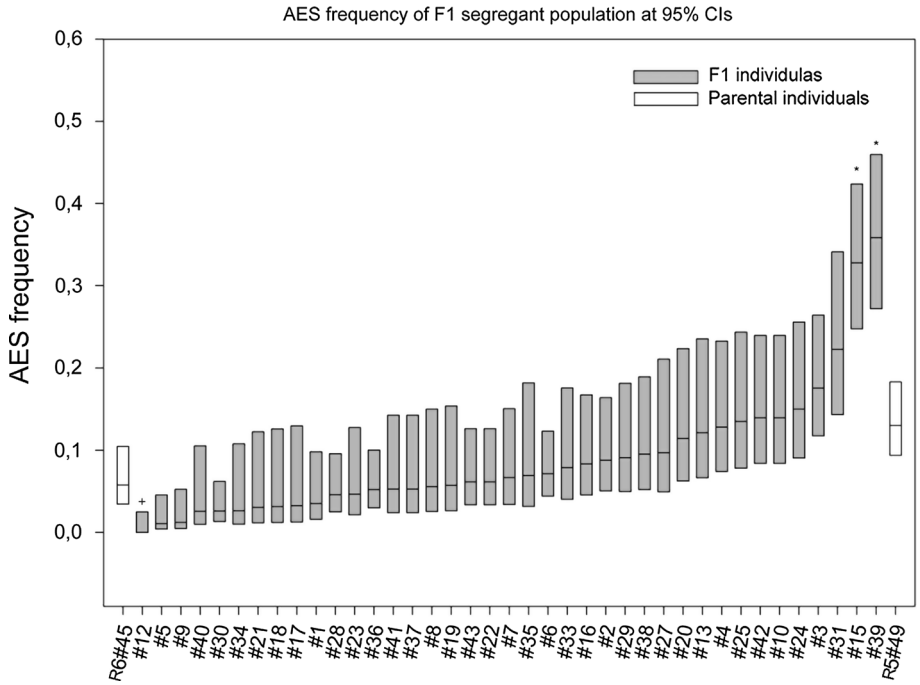

Fig. 3 Expressivity of apospory in diploid genotypes of *Paspalum rufum* and its F_1 progeny. The frequency of ovaries containing AES scored in each plant is depicted together with 95 % confidence intervals (CIs). Parental genotypes (*white box*) and F_1 individuals (*grey box*) are indicated in the *X*-axis. *Plus* indicates significant differences at $p < 0.05$ with respect to the maternal R6#45 genotype, and *asterisk* indicates significant differences with respect to the paternal R5#49 genotype

Table 3 Generation of tetraploid plants by colchicine treatment (0.1 % w/v for 6 h) of *Paspalum rufum* caryopses from R6#45

		Number of		
		Caryopses	Surviving seedlings	Tetraploid plants recovered
Open pollination	Control	15	8	0
	Colchicine	50	5	1
R6#45 × R2#18	Control	20	6	0
	Colchicine	100	5	1

experimental cross. This can be explained by the fact that most of the ovules carrying AES also contained a MES, which is always well-localized in front of the micropylar end. This spatial organization could favour the fertilization of the MES to the detriment of the aposporous ones. Moreover, an important role could be assigned to the formation of the endosperm. It is well-known that a 2:1 DNA maternal/paternal genomic ratio is an important constraint for sexual seeds development (Birchler 1993). Any deviation from this rule inhibits seed formation and is considered the primary cause of seed abortion in inter-ploidy and inter-specific crosses (Quarin 1999). In the case of double fertilization of AES ($2n$) by reduced pollen nuclei from a diploid, a seed with a DNA maternal/paternal contribution

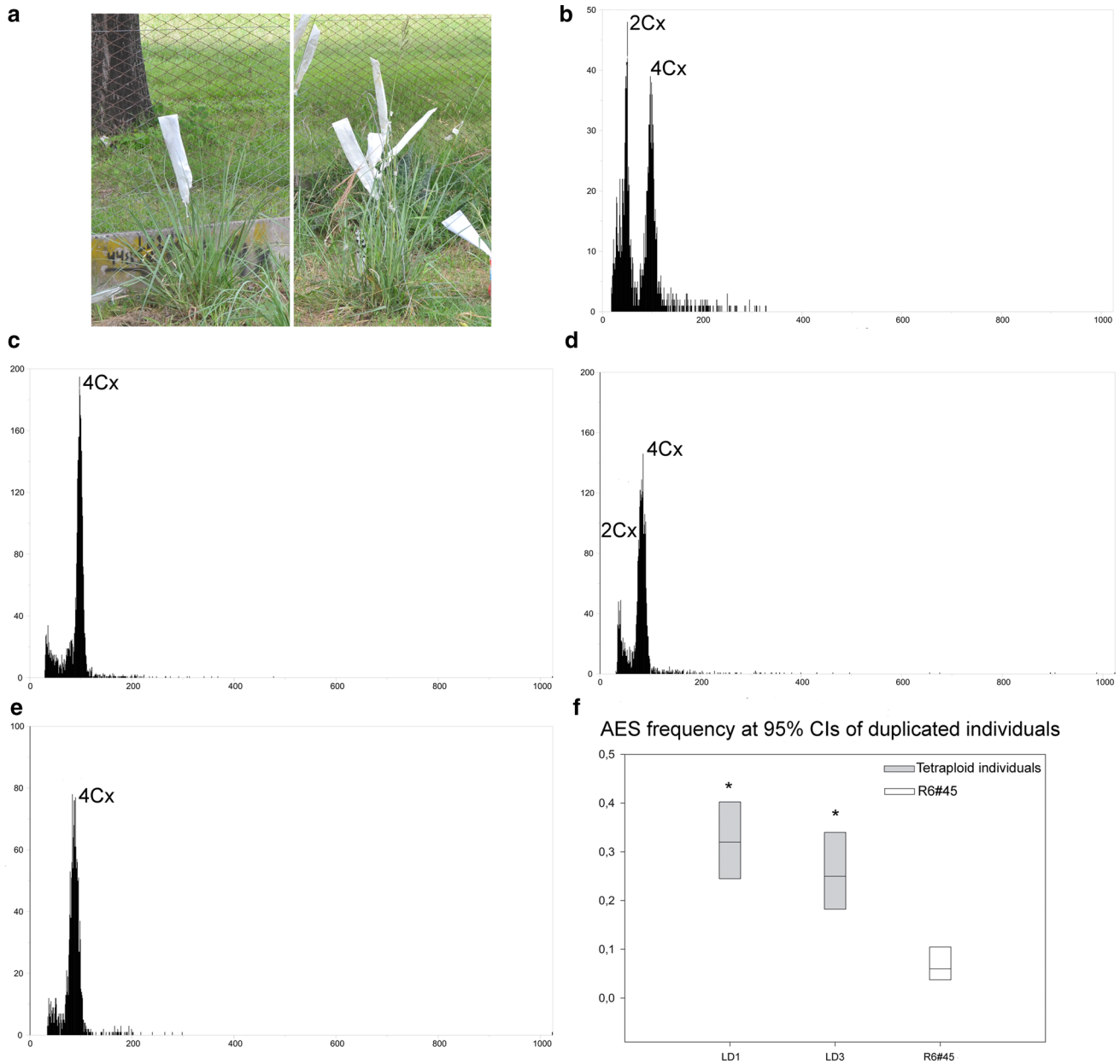

Fig. 4 Colchicine-induced autotetraploid genotypes of *Paspalum rufum*. **a** Duplicated plants LD1 (*left*) and LD3 (*right*) growing in the field, **b**, **d** histograms (in linear scale) of DNA content of leaves from LD1 (**b**) and LD3 (**d**) in the presence of a diploid internal standard, **c** LD1 and **e** LD3 histograms without internal control to confirm the homoploid condition of the experimental plant, **f** graph plot showing the frequency of ovaries carrying AES and their 95 % CIs in colchicine-duplicated individuals (LD1 and LD3) and R6#45. *Asterisk* indicates significant differences with respect to R6#45. Cx denotes monoploid DNA content

of 4:1 to the endosperm will be generated. This ratio would be non-functional in a diploid environment. However, some diploid genotypes of *P. rufum* were able to produce B_{III} hybrids, S_{III} and maternal seeds from AES in interploidy crosses with pollen from a tetraploid ($n = 2x$) (Delgado et al. 2014).

Cytoembryological analysis of F_1s showed that almost all hybrids inherited apospory capacity. Only one individual was free (or produced a very low frequency) of AES. The high proportion of individuals expressing the trait suggested that apospory is controlled by a multiallelic locus or, alternatively, by multiple loci. Genetic analyses of the inheritance of apospory in tetraploid *Paspalum simplex* and *P. notatum* indicated that it segregated as a single dominant locus (with tetrasomic or disomic behaviour), with distorted segregation ratios (Martínez et al. 2001; Pupilli et al. 2001). In most cases, distortion in segregation favoured the sexual progenies against the apomictic ones

Table 4 Cytoembryological analysis of ovules from duplicated ($2n = 4x = 40$) plants LD1 and LD3 of *P. rufum*

Plant	Ovules scored						% AES[a]
	Total	AbES	MES	AES	MES + AES	NC	
LD1	72	9	40	0	23	0	31.9
LD3	61	1	45	4	11	0	24.6

AbES ovaries containing aborted or non-well-developed embryo sacs, *MES* ovaries containing meiotic embryo sacs, *AES* ovaries containing aposporous embryo sacs, *MES + AES* ovaries containing a meiotic and one or two aposporous embryo sacs, *NC* ovules non-classified

[a] *% AES* percentage of ovaries carrying aposporous embryo sacs over the total ovaries scored

(Acuña et al. 2011; Martínez et al. 2001; Stein et al. 2004; Zilli et al. 2015). Similar results were obtained in species of the Plicatula group of *Paspalum* (Aguilera et al. 2011, 2015). The association with some pleiotropic lethal effect or with a partial lethality factor/s linked to apospory determinants was proposed to explain the distortion observed (Martínez et al. 2001; Pupilli et al. 2001). Interestingly, in *P. notatum*, the presence of a major chromosome rearrangement (such as an inversion or translocation) associated with the transmission of apospory has been reported by Podio et al. (2012). Moreover, genetic analysis revealed that the apospory-controlling region in *P. simplex* and *P. notatum* is characterized by a strong suppression in recombination (Pupilli et al. 2004; Stein et al. 2004). According to these antecedents, a multiallelic locus, rather than multiple loci, is the most probable explanation for results obtained in diploid *P. rufum.* This hypothesis should still be proved by analysing BC_1 and other segregating populations. On the other hand, expressivity analysis in hybrids showed a wide range of variation, but most plants fell within the range displayed by the parental plants. Interestingly, the variation in the expressivity was almost continuous between 0 and 15 %, but several individuals showing extreme values were also detected (with expressivity values rising up to 35.85 %). In the single multiallelic model, this variation could be explained by the combination of different alleles segregating in the population and the existence of a superior allele combination that enhanced the expression of the trait. Thus, after crossing two diploid genotypes with relatively low proportions of AES, it was possible to recover hybrids with middle and high expressions of the trait. These genotypes could be used in new crosses in order to further increase the expression of the character at the diploid level in the species. In the *Boechera* system (after a large number of accessions had been screened), besides obligate sexual plants, three levels of apomeiotic expression (low, high, and obligate) were reported. Interestingly, no intermediate genotypes with a mean apomeiosis level of 3–87 % were detected (Aliyu et al. 2010). Authors explained these results by considering that both obligate sexual and low-frequency apomeiosis accessions are sensu stricto facultative apomictic, but at the population-level dynamics (e.g. gene flow), they likely reflect sexuality rather than apomixis. Although this hypothesis does not explain the absence of intermediate apomeiosis frequencies, it may be correlated with the complex gene regulatory changes associated with apomeiosis (Aliyu et al. 2010; Sharbel et al. 2009, 2010).

Analysis of apospory expressivity in synthetic autotetraploids showed that polyploidization significantly increases the proportion of AES compared to the original genotype. Moreover, expressivity values were comparable to the extreme phenotypes obtained in the diploid F_1 population. This outcome confirmed the role of ploidy in the expression of apomixis and demonstrated that duplication of the genome complement increased the capacity for apospory, probably due to the increase in favourable allele dosage. Previous works in *Paspalum* spp. have shown that colchicine-duplicated individuals from sexual diploids can result in completely sexual or facultative apomictic individuals (Quarin et al. 1998, 2001; Sartor et al. 2009). These studies suggested that although the genetic factor(s) for apospory may exist in diploid plants, its expression is restrained, and only after doubling of the chromosome number, some kind of dosage effect increases its expression (Quarin et al. 2001).

Due to the absence of an evident expression of apomixis in diploid grasses, analyses of the inheritance of the trait at this ploidy level are still lacking (Pupilli and Barcaccia 2012). Our study proposes an innovative model to analyse apospory and apomixis in a diploid genome environment. The fact that more than one allele/gene controlling apospory could be segregating in diploids opens the possibility for disclosing the genetic components underlying the trait. On the other hand, as apomictic individuals are frequently facultative, both sexual and apomictic pathways coexist to different extensions in the same genotype, suggesting that factors that are still unknown are conditioning its penetrance and expressivity (Asker and Jerling 1992; Spillane et al. 2001). Recent studies in tetraploid *P. notatum* have found that apomixis expressivity presents a high level of variation between full-sib hybrids derived from crosses between sexual and apomictic genotypes (Acuña et al. 2011; Zilli et al. 2015). Studies in *Poa* have postulated that apospory and parthenogenesis are controlled by different genetic pathways, that more than one gene is involved in each character regulation, and that the interaction between the different genes involved would induce different expressivity of the character (Matzk et al. 2005). These antecedents support the hypothesis that expressivity of apomixis can be genotypic dependent and suggest that other factors, possibly

acting through dosage effects, could influence the trait (Acuña et al. 2011; Aliyu et al. 2010; Matzk et al. 2005; Schranz et al. 2005; Zilli et al. 2015). Despite its high agronomical importance, the control of apomixis components expressivity is far from being understood (Spillane et al. 2001). Interesting advances have been made to generate new apomictic genotypes, but the frequency of highly apomictic individuals is lower than expected for a single dominant Mendelian factor, and only low proportions of them were recovered after different experiments (Acuña et al. 2011; Zilli et al. 2015). The determination of the control of apomixis components could help to express this important trait in a diploid environment.

Acknowledgments This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina (PICT 2011–1269 and PICT 2014–1080); Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina [grant no. PIP 11220090100613, PIP 114201101–00283]; Fundación Ciencias Agrarias (PICT-2015). L.D., M.E.S., F.E., M.S., and J.P.A.O. are staff members of CONICET, Argentina.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Information on Electronic Supplementary Material

Online Resource 1. UBC primer screened to detect polymorphism between parental genotypes (R6#45 and R5#49) or segregation in F_1 population.
Online Resource 2. Examples of cytometric histograms (in logarithmic scale) of leaves from F_1 progenies (R6#45 x R5#49) in the presence of a diploid internal control.

References

Acuña C, Blount AR, Quesenberry KH, Kenworthy KE, Hanna WW (2011) Tetraploid bahiagrass hybrids: breeding technique, genetic variability and proportion of heterotic hybrids. Euphytica 179:227–235. doi:10.1007/s10681-010-0276-y

Aguilera PM, Sartor ME, Galdeano F, Espinoza F, Quarin CL (2011) Interspecific tetraploid hybrids between two forage grass species: sexual and apomictic. Crop Sci 51:1544–1550. doi:10.2135/cropsci2010.10.0610

Aguilera PM, Galdeano F, Quarin CL, Ortiz JPA, Espinoza F (2015) Inheritance of aposporous apomixis in inter-specific hybrids derived from sexual and apomictic. Crop Sci 55:1947–1956. doi:10.2135/cropsci2014.11.0770

Aliyu OM, Schranz ME, Sharbel TF (2010) Quantitative variation for apomictic reproduction in the genus *Boechera* (Brassicaceae). Amer J Bot 97:1719–1731. doi:10.3732/ajb.1000188

Asker SE, Jerling L (1992) Apomixis in plants. CRC Press, Boca Raton

Bennett HW, Bashaw EC (1966) Interspecific Hybridization with *Paspalum* spp. Crop Sci 6:52–54. doi:10.2135/cropsci1966.0011183X000600010016x

Birchler JA (1993) Dosage analysis of maize endosperm development. Annual Rev Genet 27:181–204. doi:10.1146/annurev.ge.27.120193.001145

Burson BL (1991) Genome relationships between tetraploid and hexaploid biotypes of dallisgrass, *Paspalum dilatatum*. Bot Gaz 152(2):219–223

Carman JG (1997) Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. Biol J Linn Soc 61:51–94. doi:10.1111/j.1095-8312.1997.tb01778.x

Delgado L, Galdeano F, MaE Sartor, Quarin CL, Espinoza F, Ortiz JP (2014) Analysis of variation for apomictic reproduction in diploid *Paspalum rufum*. Ann Bot (Oxford) 113:1211–1218. doi:10.1093/aob/mcu056

Espinoza F, Daurelio LD, Pessino SC, Valle EM, Quarin CL (2006) Genetic characterization of *Paspalum notatum* accessions by AFLP markers. Pl Syst Evol 258:147–159. doi:10.1007/s00606-005-0401-x

Hanna WW, Bashaw EC (1987) Apomixis: its identification and use in plant breeding. Crop Sci 27:1136–1139. doi:10.2135/cropsci1987.0011183X002700060010x

Hojsgaard D, Schegg E, Valls JFM, Martínez EJ, Quarin CL (2008) Sexuality, apomixis, ploidy levels, and genomic relationships among four *Paspalum* species of the subgenus *Anachyris* (*Poaceae*). Flora Morphol Distrib Funct Ecol Plants 203:535–547. doi:10.1016/j.flora.2007.09.005

Martínez EJ, Urbani MH, Quarin CL, Ortiz JP (2001) Inheritance of apospory in bahiagrass, *Paspalum notatum*. Hereditas 135:19–25. doi:10.1111/j.1601-5223.2001.00019.x

Matzk F, Prodanovic S, Baumlein H, Schubert I (2005) The Inheritance of apomixis in *Poa pratensis* confirms a five locus model with differences in gene expressivity and penetrance. Pl Cell 17:13–24. doi:10.1105/tpc.104.027359

MeasuringU (2012) MeasuringU. A/B Test Calculator-N-1 Two Proportion test for comparing independent proportions for small and large sample sizes. http://www.measuringu.com/ab-calc.php

Newcombe RG (1998) Two sided confidence intervals for the single proportion: comparison of seven methods. Statist Med 17:857–872. doi:10.1002/(SICI)1097-0258(19980430)17:8<857:AID-SIM777>3.0.CO;2-E

Nogler GA (1984) Gametophytic apomixis. In: Johri BM (ed) Embryology of angiosperms. Springer, Berlin, pp 475–518

Norrmann GA, Quarin CL, Burson BL (1989) Cytogenetics and reproductive behavior of different chromosome races in six *Paspalum* species. J Heredity 80:24–28

Ortiz JP, Quarin CL, Pessino SC, Acuña C, Martínez EJ, Espinoza F, Hojsgaard DH, Sartor ME, Cáceres ME, Pupilli F (2013) Harnessing apomictic reproduction in grasses: what we have learned from *Paspalum*. Ann Bot (Oxford) 112:767–787. doi:10.1093/aob/mct152

Podio M, Siena LA, Hojsgaard D, Stein J, Quarin CL, Ortiz JPA (2012) Evaluation of meiotic abnormalities and pollen viability in aposporous and sexual tetraploid *Paspalum notatum* (*Poaceae*). Pl Syst Evol 298:1625–1633. doi:10.1007/s00606-012-0664-y

Pupilli F, Barcaccia G (2012) Cloning plants by seeds: inheritance models and candidate genes to increase fundamental knowledge for engineering apomixis in sexual crops. J Biotechnol 154:291–311. doi:10.1016/j.jbiotec.2011.08.028

Pupilli F, Caceres ME, Arcioni S, Quarin CL (1997) Segregation analysis of RFLP markers reveals a tetrasomic inheritance in apomictic *Paspalum simplex*. Genome 40:822–828. doi:10.1139/g97-806

Pupilli F, Labombarda P, Cáceres ME, Quarn CL, Arcioni S (2001) The chromosome segment related to apomixis in *Paspalum simplex* is homoeologous to the telomeric region of the long arm

of rice chromosome 12. Molec Breed 8:53–61. doi:10.1023/A:1011966922301

Pupilli F, Martínez EJ, Busti A, Calderini O, Quarin CL, Arcioni S (2004) Comparative mapping reveals partial conservation of synteny at the apomixis locus in *Paspalum* spp. Molec Genet Genomics 270:539–548. doi:10.1007/s00438-003-0949-5

Quarin CL (1986) Seasonal changes in the incidence of apomixis of diploid, triploid, and tetraploid plants of *Paspalum cromyorrhizon*. Euphytica 35:515–522. doi:10.1007/BF00021860

Quarin CL (1992) The nature of apomixis and its origin in Panicoid grasses. Apomixis Newsl 5:8–15

Quarin CL (1999) Effect of pollen source and pollen ploidy on endosperm formation and seed set in pseudogamous apomictic *Paspalum notatum*. Sexual Pl Reprod 11:331–335. doi:10.1007/s004970050160

Quarin CL, Norrmann GA (1987) Cytology and reproductive behavior of *Paspalum equitans*, *P. ionanthum*, and their hybrids with diploid and tetraploid cytotypes of *P. cromyorrhizon*. Bot Gaz 148:386–391. doi:10.1086/337667

Quarin CL, Burson BL, Burton GW (1984) Cytology of intra-and interspecific hybrids between two cytotypes of *Paspalum notatum* and *P. cromyorrhizon*. Bot Gaz 3:420–426. doi:10.1086/337474

Quarin CL, Norrmann GA, Espinoza F (1998) Evidence for autoploidy in apomictic *Paspalum rufum*. Hereditas 129:119–124. doi:10.1111/j.1601-5223.1998.00119.x

Quarin CL, Espinoza F, Martinez EJ, Pessino SC, Bovo OA (2001) A rise of ploidy level induces the expression of apomixis in *Paspalum notatum*. Sexual Pl Reprod 13:243–249. doi:10.1007/s004970100070

Sartor ME, Quarin CL, Espinoza F (2009) Mode of reproduction of colchicine-induced *Paspalum plicatulum* tetraploids. Crop Sci 49:1270–1276. doi:10.2135/cropsci2008.05.0270

Sartor ME, Quarin CL, Urbani MH, Espinoza F (2011) Ploidy levels and reproductive behaviour in natural populations of five *Paspalum* species. Pl Syst Evol 293:31–41. doi:10.1007/s00606-011-0416-4

Savidan Y (2000) Apomixis: genetics and breeding. Pl Breed Rev 18:13–86. doi:10.1002/9780470650158.ch2

Schranz ME, Dobes C, Koch MA, Mitchell-Olds T (2005) Sexual reproduction, hybridization, apomixis, and polyploidization in the genus *Boechera* (Brassicaceae). Amer J Bot 92:1797–1810. doi:10.3732/ajb.92.11.1797

Sharbel TF, Voigt ML, Corral JM, Thiel T, Varshney A, Kumlehn J, Vogel H, Rotter B (2009) Molecular signatures of apomictic and sexual ovules in the *Boechera holboellii* complex. Pl J 58:870–882. doi:10.1111/j.1365-313X.2009.03826.x

Sharbel TF, Voigt ML, Corral JM, Galla G, Kumlehn J, Klukas C, Schreiber F, Vogel H, Rotter B (2010) Apomictic and sexual ovules of *Boechera* display heterochronic global gene expression patterns. Pl Cell 22:655–671. doi:10.1105/tpc.109.072223

Siena LA, Sartor ME, Espinoza F, Quarin CL, Ortiz JPA (2008) Genetic and embryological evidences of apomixis at the diploid level in *Paspalum rufum* support recurrent auto-polyploidization in the species. Sexual Pl Reprod 21:205–215. doi:10.1007/s00497-008-0080-1

Speranza PR (2009) Evolutionary patterns in the Dilatata group (*Paspalum*, *Poaceae*). Pl Syst Evol 282:43–56. doi:10.1007/s00606-009-0205-5

Spillane C, Steimer A, Grossniklaus U (2001) Apomixis in agriculture: the quest for clonal seeds. Sexual Pl Reprod 14:179–187. doi:10.1007/s00497-001-0117-1

Stein J, Quarin CL, Martínez EJ, Pessino SC, Ortiz JPA (2004) Tetraploid races of *Paspalum notatum* show polysomic inheritance and preferential chromosome pairing around the apospory-controlling locus. Theor Appl Genet 109:186–191. doi:10.1007/s00122-004-1614-z

Upton GJ (1992) Fisher's exact test. J Roy Stat Soc Ser A (Stat Soc) 155:395–402. doi:10.2307/2982890

VassarStats (2016) VassarStats: Website for Statistical computation. Available at: http://vassarstats.net/

Wilson EB (1927) Probable inference, the law of succession, and statistical inference. J Amer Statistical Association 22:209–212. doi:10.1080/01621459.1927.10502953

Young BA, Sherwood RT, Bashaw EC (1979) Cleared-pistil and thick-sectioning techniques for detecting aposporous apomixis in grasses. Canad J Bot 57:1668–1672. doi:10.1139/b79-204

Zilli AL, Brugnoli EA, Marcón F, Billa MB, Rios EF, Martínez EJ, Acuña CA (2015) Heterosis and expressivity of apospory in tetraploid bahiagrass hybrids. Crop Sci 55:1189–1201. doi:10.2135/cropsci2014.10.0685

Zuloaga FO, Morrone O (2005) Revisión de las especies de *Paspalum* para América del Sur austral (Argentina, Bolivia, sur del Brasil, Chile, Paraguay y Uruguay). Monogr Syst Bot Missouri Bot Gard 102:1–297