Inheritance of Aposporous Apomixis in Inter-specific Hybrids Derived from Sexual *Paspalum plicatulum* and Apomictic *Paspalum guenoarum*

Patricia Mabel Aguilera,★ Florencia Galdeano, Camilo Luis Quarin, Juan Pablo Amelio Ortiz, and Francisco Espinoza

ABSTRACT

Apomictic *Paspalum plicatulum* Michx. and *P. guenoarum* Arechav. are promising candidates for forage grass development in tropical regions. From a plant breeding perspective, apomixis provides a unique mechanism for developing superior cultivars and preserving them indefinitely. Therefore, analysis of its inheritance is of great interest in these apomictic species. The objective of this work was to analyze the inheritance and expressivity of apomixis in interspecific families derived from crosses between a sexual colchicine-induced plant of *P. plicatulum* and an apomictic *P. guenoarum* plant. One F_1, one F_2, and three backcross (BC) populations were created. Amplified fragment-length polymorphism (AFLP) markers confirmed the hybrid origin of the F_1 descendants. Analysis of the reproductive mode of the F_1 by flow cytometry seed screen (FCSS) showed a segregation ratio of 1.6:1 sexual vs. apomictic plants. The same analysis in F_2 and BC populations showed that in selfing or crosses involving sexual genotypes all progeny reproduced by sexuality, while BC populations involving apomictic hybrids as pollen donors produced offspring that segregated for the reproductive mode. Apomixis can be transmitted by pollen from *P. guenoarum* to other species of the Plicatula group as a simplex (Aaaa) Mendelian dominant factor, and full sexual reproduction requires the homozygous recessive (nulliplex) condition (aaaa) for the apomixis determinant. Results provided here are of interest for breeding apomictic species of Plicatula and for basic research on the genetic determinants of apomixis. Also, the possibility of creating new apomictic cultivars by traditional breeding methods is presented.

P.M. Aguilera, F. Galdeano, C.L. Quarin, J.P. Amelio Ortiz, and F. Espinoza, Instituto de Botánica del Nordeste (IBONE-CONICET), Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste (UNNE), Casilla de Correo 209, (3400), Corrientes, Argentina; P.M. Aguilera, Instituto de Biología Subtropical, Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones (IBS-UNaM-CONICET), (3300), Posadas, Misiones, Argentina; J.P. Amelio Ortiz, Laboratorio de Biología Molecular, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario (UNR), Casilla de Correo 14 (S2125ZAA), Zavalla, Santa Fe, Argentina. Received 17 Nov. 2014. Accepted 18 Mar. 2015. ★Corresponding author (patriciamaguilera@gmail.com).

Abbreviations: ACL, apomixis controlling locus; AFLP, amplified fragment-length polymorphism; BC, backcross; FCSS, flow cytometry seed screen.

Apomixis in angiosperms means asexual (agamic) reproduction by seeds. This mode of reproduction leads to maternal offspring that normally are genetically identical copies of the mother plant (Nogler, 1984). Apomixis has been observed in 223 genera (of about 14,000), 41 of which belong to the family Poaceae (Carman et al., 2011). Apomictic reproduction entails the development of an embryo from a cell with a somatic chromosome number. It involves two fundamentally different pathways: adventitious embryony (i.e., sporophytic apomixis) in which maternal embryos develop directly from somatic cells of the ovule; and gametophytic apomixis in which female gametophyte arises from an unreduced initial cell. According to the origin of the embryo, gametophytic apomixis can be distinguished in diplospory, in which the unreduced embryo sac originates from the megaspore mother cell itself either directly by mitosis or

indirectly by a failure in meiosis, and apospory, in which the unreduced embryo sac originates from somatic cells of the ovule (usually from the nucellus) (Nogler, 1984).

Apomixis is a heritable trait thought to have evolved through a rearrangement of the developmental programs that constitute the normal sexual pathway (Grimanelli et al., 2001). From a plant breeding perspective, apomixis provides a unique mechanism for developing superior cultivars and preserving them indefinitely (Ortiz et al., 2013). Introduction of apomixis into agronomically important crops would allow full exploitation of heterosis by reseeding elite hybrids and clonal propagation of superior genotypes in seed-propagated outcrossing crops. Unfortunately, with a few exceptions in some forage grasses and fruit trees, apomixis is not a common feature among crop species (Barcaccia and Albertini, 2013).

In grasses, the most widely accepted genetic model for the inheritance of apomixis postulates that the trait is controlled by a single dominant locus (Savidan, 2000). In guineagrass (*Panicum maximum* Jacq.) (Savidan, 1982), *Brachiaria* hybrids (Valle and Glenke, 1993), buffelgrass (*Cenchrus ciliaris* L.) (Sherwood et al., 1994; Jessup et al., 2002), and Kentucky bluegrass (*Poa pratensis* L.) (Barcaccia et al., 1998), apomixis has shown to be controlled by one or a few linked Mendelian factors. The observed segregation pattern for the reproductive mode was equal or not significantly different from 1:1 (sexual vs. apomictic) in these species. However, a strong segregation distortion has been observed for the trait in other grass species such as maize–*Tripsacum* hybrids (Grimanelli et al., 1998), *Pennisetum* spp. (Ozias-Akins et al., 1998; Roche et al., 2001) and *Paspalum* spp. (Martínez et al., 2001; Pupilli et al., 2001; Acuña et al., 2009; Hojsgaard et al., 2011). In these systems, segregation ratios of sexuality vs. apomixis differed from the expected 1:1, favoring sexual individuals. Moreover, molecular studies of the apomixis controlling locus (ACL) in several of these species have revealed that it comprises a large chromosome segment involving a lack of recombination, presence of repetitive elements, and epigenetic modifications (Pupilli and Barcaccia, 2012).

In particular, the genus *Paspalum* L. constitutes a very interesting system to study apomixis because it is both an important target crop and a model system for mining candidate genes (Ortiz et al., 2013). Most apomictic *Paspalum* species form aposporous embryo sacs from somatic (2n) cells of the nucellus, regardless of whether meiosis occurs in the megaspore mother cell of the same ovule or not. However, frequently, the four megaspores degenerate after meiosis when active aposporous initial cells are present in an ovule. Diplospory occurs less frequently in the genus (Quarin, 1992; Ortiz et al., 2013). The two archetypal model species within *Paspalum* spp., *P. notatum* Flüggé (bahiagrass), and *P. simplex* Morong, led to the more advanced results concerning apomixis inheritance in the genus (Ortiz et

al., 2013). Evaluations performed on different F_1 populations of *P. notatum* (derived from crosses between experimentally obtained completely sexual tetraploid genotypes and natural apomictic accessions) showed segregation ratios ranging from 2.8:1 to 6.5:1 (sexual vs. apomictic, both obligated and facultative) individuals (Martínez et al., 2001; Stein et al., 2004; Acuña et al., 2009). A pleiotropic lethal dominant allele with incomplete penetrance or partial lethality of factors linked to apospory may account for these distorted segregation patterns (Martínez et al., 2001). A survey of meiosis in a group of natural apomictic accessions, experimentally generated sexual genotypes and F_1 hybrids showed that apomictic strains had a genetic rearrangement that is transmitted associated with the apomictic mode of reproduction (Podio et al., 2012). Similarly, inheritance of apomixis in *P. simplex* showed a segregation ratio of 1.6:1 (sexual vs. apomictic) in a tetraploid backcross population derived from crossing a colchicine-induced sexual autotetraploid ($2n = 4x = 40$) genotype with a natural tetraploid apomict (Pupilli et al., 2001). The model of inheritance proposed for the trait in *P. simplex* assumes apomixis is controlled by a single dominant gene, with or without linkage to a recessive lethal gene.

Paspalum plicatulum and *P. guenoarum* are two perennial grass species that belong to the taxonomically informal infrageneric Plicatula group (Zuloaga and Morrone, 2005). These species naturally inhabit the South American prairies and are autotetraploids ($2n = 4x = 40$) that share the same basic genome complement and reproduce by aposporous apomixis followed by pseudogamy (Aguilera et al., 2011). Additionally, sexual diploids ($2n = 2x = 20$) were found for *P. plicatulum* as well as for other Plicatula species but not for *P. guenoarum*. Like most Plicatula members, *P. plicatulum* and *P. guenoarum* are valuable wild forage grasses in native grasslands of South America and have been introduced as forage crops in warm regions of the world (Ramirez, 1954; Oram, 1990). However, genetic improvement through conventional breeding techniques has not been accomplished, mainly because of the apomictic mode of reproduction of the tetraploid cytotypes of these species. Recently, a sexual tetraploid plant (4c-4x) was obtained by colchicine treatment of a sexual diploid cytotype of *P. plicatulum* (Sartor et al., 2009). This individual reproduced completely by sexual means, retained the high degree of self-incompatibility of the diploids, and set seeds when crossed with pollen of apomictic tetraploid *P. guenoarum*. A small F_1 progeny, obtained by crossing a clone of 4c-4x named 4PT with *P. guenoarum*, showed an intermediate phenotype and segregated for apomixis (Aguilera et al., 2011). These first results demonstrated that it was possible to exchange genes among these species and generate interspecific fertile hybrids at the tetraploid level.

The objective of this work was to analyze the inheritance and expressivity of apomixis in interspecific families
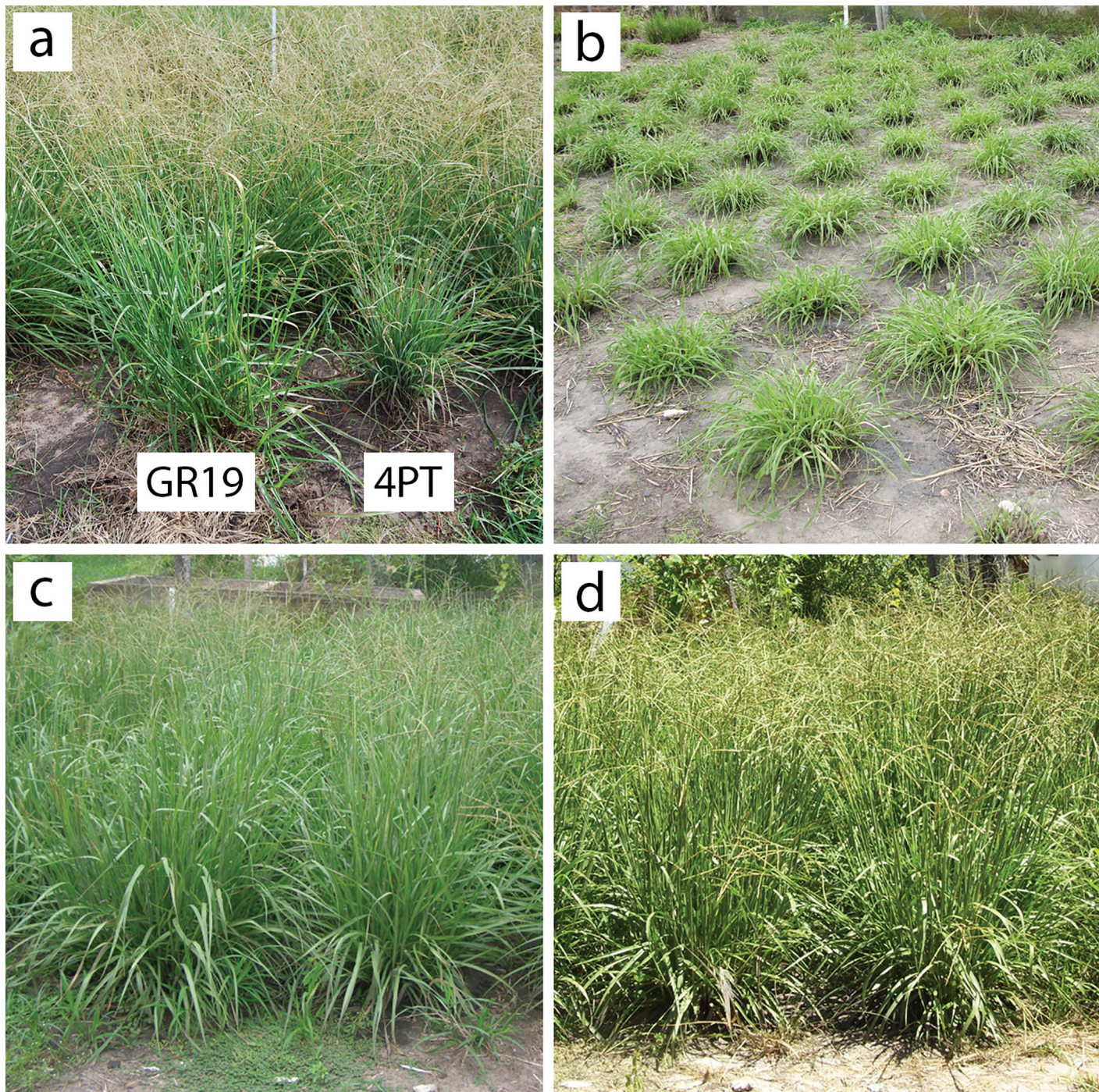
Figure 1. Parental genotypes and their F₁ hybrids. (a) GR19, the apomictic tetraploid plant of cultivar Rojas of *P. guenoarum* used as pollen donor parent, on the left, and the completely sexual tetraploid genotype of *P. plicatulum* 4PT, used as pistillate parent, on the right. (b) 4PT × GR19 F₁ individuals growing at the field a few weeks after transplanting. (c) F₁ individuals at the beginning of the flowering period. (d) F₁ individuals at the end of the flowering period.

derived from crosses between a completely sexual colchicine-induced tetraploid plant of *P. plicatulum* (4PT) and an apomictic tetraploid plant of *P. guenoarum* cv. Rojas (GR19).

MATERIALS AND METHODS
Plant Material and Crosses

An F₁ population was obtained by crossing a completely sexual tetraploid ($2n = 4x = 40$) genotype of *P. plicatulum* (4PT), used as pistillate parent, and one apomictic individual (GR19) of the tetraploid ($2n = 4x = 40$) cultivar Rojas of *P. guenoarum*, used as pollen donor parent (Fig. 1a). The genotype 4PT is a vegetatively propagated clone from genotype 4c-4x which is a tetraploid individual experimentally generated by colchicine treatment of a sexual diploid plant of *P. plicatulum* (Sartor et al., 2009). This plant retained the self-incompatibility system presented in the diploid and formed hybrids in crosses with tetraploid *P. guenoarum* (Aguilera et al., 2011). Interspecific crosses were performed following the procedure described by Aguilera et al. (2011). Four sexual F₁ plants were self-pollinated to obtain F₂

Table 1. Number of caryopses and plants generated from (i) a cross between the completely sexual tetraploid genotype 4PT of *P. plicatulum* and the natural apomictic individual GR19 from *P. guenoarum*, (ii) self-pollination of F₁ hybrids, and (iii) backcrosses between 4PT and F₁ sexual (S) and apomictic (A) individuals.

Female parent	Pollen source	No. pollinated florets	Cary-opses	Seed set	No. sown seeds	No. recovered plants
				%		
(i) Cross						
4PT S	GR19 A	1282	244	19.03	244	189
(ii) Self-pollination						
F₁#8 S	Selfed	1441	191	13.25	50	24
F₁#12 S	Selfed	529	2	0.37	–	–
F₁#29 S	Selfed	651	0	0.0	–	–
F₁#119 S	Selfed	1003	0	0.0	–	–
(iii) Backcrosses						
4PT	F₁#29 S	1398	267	19.09	30	23
4PT	F₁#15 A	529	32	6.04	32	20
4PT	F₁#62 A	911	77	8.45	30	22

populations. Moreover, crosses involving 4PT as female parent and sexual or apomictic F₁ as pollen donors were performed to obtain BC populations (Table 1). Seeds obtained after experimental crosses were collected and germinated in sterilized soil. Individual seedlings were planted in small pots, maintained in a greenhouse, transplanted to the field nursery at the Agronomy Faculty of the National University of Northeast, Corrientes, Argentina, and allowed to seed set in open pollination.

Amplified Fragment-Length Polymorphism Analysis

Amplified fragment-length polymorphism markers (Vos et al., 1995) were used to determine the hybrid origin of the F₁ plants. A random sample of 89 F₁ individuals together with both parental genotypes, 4PT and GR19, were analyzed. Genomic DNA was extracted from 0.4 g of fresh leaves following the method described by Dellaporta et al. (1983) with the modifications introduced by Ortiz et al. (1997). Sample quality controls were performed by measuring absorbance at 260 and 280 nm and by electrophoresis in 1% agarose gels to confirm DNA integrity and absence of RNA contamination. The AFLP experiments were performed using the AFLP Analysis System I (Life Technologies, Invitrogen) following the manufacturer´s instructions with the minor modifications indicated by Sartor et al. (2013). Seven primer combinations (E31M32, E31M35, E31M42, E34M32, E35M33, E35M34, and E36M32) were used in selective amplification reactions. BioRad thermocycler (MyCycler Thermal Cycler #170-9701) was used for both preamplification and selective amplification reactions. Following amplification, the polymerase chain reaction products were separated by electrophoresis on polyacrylamide gel, silver stained, and digitalized using HP Scanjet 4670 scanner (Hewlett–Packard). Individuals showing the presence of at least 10 AFLP bands segregating from the male parent were considered hybrids between 4PT and GR19.

Ploidy Level of Experimental Plants

Samples of fresh leaf tissue from 4PT × GR19 population were analyzed by flow cytometry according to Sartor et al. (2011) to determine the ploidy level of the F₁ progeny. Bulks of leaf tissue from five to 10 experimental F₁ plants plus a standard of known ploidy were prepared by mixing 0.5 cm² of leaf tissue from each plant. The previously cytogenetically studied tetraploid 4PT plant of *P. plicatulum* (Sartor et al., 2009) was used as a control. The ploidy level of F₁ plants was estimated by comparing the position of the DNA peak of the bulk sample with the DNA peak of 4PT indicating the tetraploid level. At least 3000 nuclei were counted for each sample. Additionally, the chromosome number of a random sample of F₁ hybrids was determined in root-tip cells to detect any level of aneuploidy.

Mode of Reproduction

The reproductive mode of F₁, F₂, and BC individuals was determined by using the FCSS method reported by Matzk et al. (2000) and considerations on this approach by Wieners et al. (2006) were taken into account. Because in most *Paspalum* species both meiotic and aposporous embryo sac contain two polar nuclei, the relative embryo/endosperm DNA content ratio (expressed as Cx-value, which indicates the number of repetitions of the basic number of the DNA content, in relation to the ploidy level) allows to differentiate between seeds derived from sexuality or apomixis. In a tetraploid individual ($2n = 4x = 40$), a sexually formed seed, shows an embryo/endosperm DNA content ratio of 4:6 [embryo = $n + n$; endosperm = $(n + n) + n$]. In contrast, a 4:10 ratio is expected in seed formed by apospory followed by pseudogamy [embryo = $2n + 0$; endosperm = $(2n + 2n) + n$] (Sartor et al., 2011). Accordingly, progenies producing seeds exclusively with a 4:6 DNA ratio were considered sexual, while plants producing seeds with 4:10 ratio or a mixture of 4:10 and 4:6 ratios were considered apomictic (obligated or facultative, respectively). Briefly, following open pollination, 15 to 50 freshly harvested caryopses from each F₁ plant were analyzed. Likewise, two to 22 caryopses from each F₂ or BC individuals were tested. In all cases, bulks of two to 10 caryopses per plant were chopped with a razor blade in a Petri dish containing 0.5 mL of ice-cold cell extraction buffer (CyStain UV Precise P Nuclei Extraction Buffer, Partec). Flow cytometry analyses were performed according to Aguilera et al. (2011). Moreover, to determine the expressivity of apomixis in hybrids, 35 F₁ apomictic plants were chosen at random. A minimum of 50 seeds per plant was analyzed through a seed-by-seed FCSS analysis.

Statistical Analysis

Chi-square (χ^2) test was used to compare the observed number of sexual vs. apomictic individuals with the expected ratio for a single factor in F₁, F₂, and BC populations, respectively. Only *p*-values lower than 0.05 were considered significant.

RESULTS
Generation and Analysis of F₁ Population

From a total of 1282 spikelets of 4PT dusted with pollen of GR19, 244 caryopses were recovered, yielding a seed set of 19.03% (Table 1). After germination, 189 F₁ hybrids
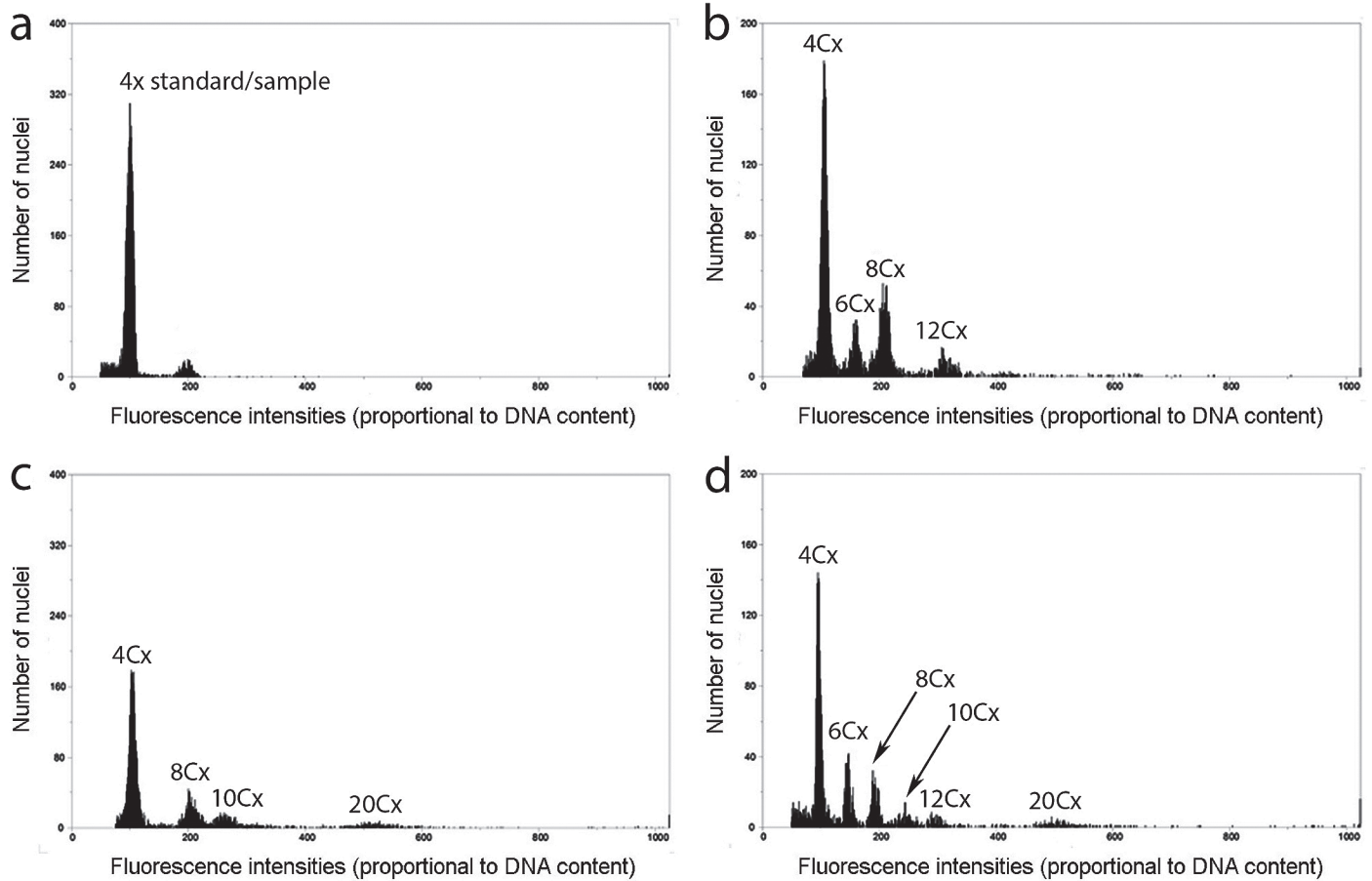
Figure 2. Flow cytometry histograms of (a) leaf tissue and (b–d) seeds of *P. plicatulum* × *P. guenoarum* F$_1$ hybrids. (a) Histogram of a bulked sample of leaves from ten F$_1$ individuals showing DNA content 2n = 4x. (b) Bulked seeds of a sexual hybrid with an embryo/endosperm DNA content ratio of 4Cx:6Cx. Additional peaks produced by embryo and endosperm nuclei at G2 stage of the cell cycle were observed. (c) Bulked seeds of an obligate apomictic hybrid with an embryo/endosperm DNA content ratio of 4Cx:10Cx. Additional peaks produced by embryo and endosperm nuclei at G2 stage of the cell cycle were observed. (d) Bulked seeds of a facultative apomictic hybrid with embryo/endosperm DNA content ratio of 4Cx:6Cx and 4Cx:10Cx corresponding to seeds originated by sexuality and apomixis, respectively. Additional peaks produced by embryo and endosperm nuclei at G2 stage of the cell cycle were observed.

(77.45%) were obtained and grown to maturity (Fig. 1b–d). Morphological characteristics of inflorescences of F$_1$ plants such as axis length, number of racemes per inflorescence, length of upper and lower racemes, and length and width of leaf blade resembled the male parent, suggesting that progeny was of hybrid origin. Notwithstanding, a large sample involving 89 individuals was screened with AFLP markers. All seven primer combinations tested produced appropriate amplification profiles. Analysis of the amplification patterns showed that the 89 individuals tested presented at least 10 bands segregating from the male parent (GR19) confirming their hybrid origin. From the total of 189 F$_1$ hybrids, 182 (96.29%) generated filled caryopses in open pollination. The remaining seven plants were sterile and only produced empty spikelets.

Because this population derived from an interspecific cross, the appearance of individuals with a nonexpected ploidy level could not be excluded. To avoid these types of individuals in further analyses, the ploidy level of the 189 F$_1$ hybrids was determined by flow cytometry assays in leaf sections. The relative DNA content of bulks of five to 10 F$_1$ plants was compared with the standard sample (the tetraploid 2n = 4x = 40 mother plant 4PT). Histograms of all bulks were coincident with the control (Fig. 2a). These results indicated that all the F$_1$ individuals were tetraploid hybrids with 2n = 4x = 40 chromosomes.

Analysis of the Mode of Reproduction of F$_1$ Hybrids

The determination of reproductive mode of the parental genotypes and the F$_1$ hybrids was performed using the FCSS method (Matzk et al., 2000). Histograms of seeds derived from the female plant 4PT and 112 F$_1$ hybrids showed exclusively peaks at 4Cx and 6Cx, which correspond to caryopses generated from the normal sexual reproduction. Two additional peaks, at 8Cx and 12Cx, corresponding to the G2 stage of the embryo and the endosperm, respectively, were usually observed (Fig. 2b). On the other hand, seeds from the male parent GR19 and 18 F$_1$ hybrids displayed histograms showing an embryo/

Table 2. Analysis of the mode of reproduction of segregating population of *Paspalum* spp. derived from crossing, selfing, and backcrossing.

Parental individuals		No. observed individuals		Expected sexual/ apomictic ratio	χ^2
Female	Male	Sexual	Apomictic		
4PT S[†]	GR19 A[‡]	112	70	1:1	9.69*
Self-pollinated F$_1$#8 S		24	0	1:0	0.00
4PT	F$_1$#29 S	23	0	1:0	0.00
4PT	F$_1$#15 A	10	10	1:1	0.00
4PT	F$_1$#62 A	8	14	1:1	1.64

* Significant at the 0.05 probability level.

[†] S, sexual plant.

[‡] A, apomictic plant.

endosperm DNA content ratio of 4:10 (Fig. 2c). These histograms were interpreted as derived from seeds originated by the parthenogenetic development of the embryo and the endosperm formation by pseudogamy from an aposporous embryo sac. Also, two additional peaks, at 8Cx and 20Cx were usually observed corresponding to the G2 stage of the embryo and the endosperm, respectively. The remaining 52 plants showed histograms with 4Cx, 6Cx, 8Cx, 10Cx, 12Cx, and 20Cx peaks (Fig. 2d). These histograms were considered as derived from bulks of caryopses containing a mixture of seeds originated by apomixis (parthenogenesis and pseudogamy) (4:10) and sexuality (4:6). Moreover, 6Cx and 12Cx peaks could be originated from occasional double fertilization of an aposporous embryo sac (B$_{III}$ hybrids).

According to this analysis, 70 F$_1$ hybrids (18 obligate apomict and 52 facultative apomict) inherited the capability to generate seeds by apomixis. To sum up, the F$_1$ population derived by crossing the sexual 4PT with apomictic GR19 consisted of 112 sexual and 70 apomictic individuals. These values represented an overall segregation ratio of 1.6:1 sexual vs. apomictic plants, which significantly differed from the 1:1 expected ratio for a single Mendelian´s factor (χ^2_{1df} = 9.69; $p < 0.05$) (Table 2).

Analysis of the Reproductive Mode of F$_2$ and Backcross Populations

To analyze the inheritance of apomixis in this experimental system, one F$_2$ and three BC populations were developed (Table 1). From a total of four sexual F$_1$ plants (#8, #12, #29, and #119) that were self-pollinated, only one (#8) produced a number of caryopses (seed set 13.25%) useful for genetic analyses (Table 1). After sowing a sample of 50 seeds, a F$_2$ population of 24 individuals was obtained. The other three F$_1$ hybrids did not generate seeds or gave a small number of them. On the other hand, the BC of 4PT with the sexual F$_1$#29 hybrid generated a seed set similar to the interspecific cross (19.09%), while backcrosses with apomictic hybrids (F$_1$#15 and F$_1$#62) showed lower values (6.04 and 8.45%). After sowing seeds from each cross (4PT

× F$_1$#29S, 4PT × F$_1$#15A, and 4PT × F$_1$#62A) three BC populations of 23, 20, and 22 individuals, respectively, were obtained (Table 1).

The FCSS analysis of F$_2$ plants, as expected, showed that all individuals reproduced exclusively by sexuality (Table 2). A similar result was obtained after analyzing the BC population derived from 4PT and sexual F$_1$#29 (Table 2), which showed that all 23 individuals formed seed by sexual reproduction. In both cases, observed segregation ratios fit the ratio 1:0 (sexual/apomictic), as expected for crosses between sexual parents without capability for apomixis. On the other hand, analysis of the BC populations derived from crosses with apomictic F$_1$ hybrids showed segregation for the reproductive mode. Sexual vs. apomictic ratios observed in both BCs (4PT × F$_1$#15A and 4PT × F$_1$#62A) did not show differences from the 1:1 expected ratio (Table 2).

Analysis of Apomixis Expressivity in Interspecific Hybrids

Since the FCSS analysis of F$_1$ hybrids showed that most of them were facultative apomicts, an estimation of the expressivity of apomixis (measured as the proportion of apomixis-derived seeds over the total seeds analyzed) was performed by testing individual caryopses in a random sample of 35 apomictic F$_1$ hybrids (Table 3). A minimum of 50 caryopses was analyzed per plant. Interestingly, all hybrids showed a high proportion of seeds derived from apomixis, with an average of 96.57% (±3.03; ranging from 88.46 to 100%). Nine hybrids were obligate apomict and 11 were facultative (with a proportion of sexual seed development ranging from 1.96 to 11.54%). Seven highly apomictic plants formed B$_{III}$ ($2n + n$) progenies through the double fertilization of aposporous embryo sacs (apospory but not parthenogenesis), and eight produced a mixture of seed formation pathways: sexuality, apospory plus parthenogenesis and pseudogamy, and apospory plus double fertilization. The highest expression of sexuality was observed in the facultative apomictic hybrid #44, which showed 11.54% of its seeds developed through the sexual pathway.

DISCUSSION

Tetraploid cytotypes of *P. guenoarum* and *P. plicatulum* are promising candidates for forage development in tropical regions of the world (Evers and Burson, 2004). However, despite their high agronomic importance and because of their natural apomictic mode of reproduction, the introduction of desirable traits by conventional breeding has not been applied so far. In this context, analysis of the inheritance of apomixis, one of the key traits for releasing new cultivars, is of great interest. Moreover, it can help to identify genes controlling the character, which are essential for engineering apomixis in sexual crops. In this regard, generation of large segregating populations is an important step toward understanding the inheritance of this trait.

Table 3. Analysis of apomixis expressivity in 35 F$_1$ hybrids by flow cytometry seed screen of single seeds.

Hybrid (#)	No. seeds analyzed	Percentage of seeds originated by		
		Sexuality ($n + n$)	Apomixis ($2n + 0$)	B$_{III}$[†] ($2n + n$)
4	55	0	98.18	1.82
6	50	0	100	0
10	51	0	100	0
15	51	0	98.04	1.96
23	52	0	98.08	1.92
31	50	0	100	0
33	56	3.57	96.43	0
34	68	2.94	91.18	5.88
44	52	11.54	88.46	0
45	50	2	96	2
52	50	0	100	0
53	50	6	92	2
58	59	3.38	96.62	0
62	50	0	100	0
63	50	4	92	4
66	50	8	92	0
68	50	6	94	0
72	51	0	100	0
74	53	3.77	96.23	0
82	50	4	96	0
83	50	4	96	0
85	51	0	98.04	1.96
86	54	0	100	0
93	50	0	100	0
96	53	3.77	96.23	0
97	51	3.92	94.12	1.96
98	52	0	98.07	1.93
99	50	2	94	4
100	50	0	98	2
103	51	1.96	94.11	3.93
106	50	4	94	2
112	50	0	100	0
113	52	3.84	96.16	0
115	50	2	98	0
117	50	0	98	2

[†] B$_{III}$, hybrids originated through the fertilization of an unreduced female gamete ($2n$) with a reduced male gamete (n).

In this work, we analyzed the inheritance of apomixis in several populations (Table 1) derived from an initial interspecific cross between a sexual tetraploid plant (4PT) of *P. plicatulum* and a tetraploid apomictic genotype (GR19) of *P. guenoarum* 'Rojas'. Using flow cytometry we measured the mode of reproduction of F$_1$, F$_2$, and BC individuals and the proportion of seeds produced via apomixis (apospory plus parthenogenesis) and sexuality (meiosis plus double fertilization). As previously reported for different species of the genus (Siena et al., 2008; Sartor et al., 2009, 2011; Aguilera et al., 2011; Rebozzio et al., 2011; Hojsgaard et al., 2013), the FCSS method allowed us to rapidly and easily determine the reproductive mode of a large number of plants.

Analysis of reproductive mode of the F$_1$ showed an overall segregation ratio of 1.6:1 sexual vs. apomictic plants. This proportion clearly represents a deviation from the 1:1 ratio (Table 2), expected for a trait controlled by a single Mendelian allele segregating from a tetraploid genotype with polysomic or disomic inheritance, which is the most widely accepted genetic model for the inheritance of apomixis in grasses (Savidan, 2000). Moreover, segregation analysis of the mode of reproduction in F$_2$ and BC populations (Table 2) showed that in selfing or crosses involving sexual genotypes (i.e., F$_1$#8 or 4PT × F$_1$#29) all progeny reproduced sexually. These outcomes indicated that sexual genotypes (4PT, F$_1$#8 and F$_1$#29) carried null alleles for apomixis and only have the capacity for sexual reproduction. On the other hand, the BC populations (4PT × F$_1$#15 and 4PT × F$_1$#62) involving apomictic hybrids as pollen donors produced offspring that segregated for the reproductive mode. The observed ratio between sexual and apomictic descendants in both crosses did not differ significantly from 1:1 (Table 2). Interestingly, segregation proportion of BC 4PT × F$_1$#15 also fits to the segregation ratio observed in the F$_1$ ($\chi^2_{1.6:1}$ = 1.13), but unexpectedly BC 4PT × F$_1$#62 differed significantly from it ($\chi^2_{1.6:1}$ = 5.89). Since a number of sexual plants exceeding the number of apomictic descendants that was observed in F$_1$, it would be expected that this distortion remains constant in backcrosses and through hybridization cycles (Acuña et al., 2011). As this was not the case for both BC populations analyzed here, further studies with larger populations are needed to explain this apparently inconsistent behavior.

Results obtained in this work demonstrate that (i) apomixis can be transmitted by pollen from *P. guenoarum* to other species of the Plicatula group, (ii) the trait is inherited or transmitted as a simplex (Aaaa) Mendelian dominant factor, and (iii) full sexual reproduction requires the homozygous recessive (nulliplex) condition (aaaa) for the apomixis determinant.

Similar inheritance models of apomixis were proposed for other *Paspalum* species in which an excess of sexual progeny was observed (Martínez et al., 2001; Pupilli et al., 2001, 2004; Acuña et al., 2009, 2011; Hojsgaard et al., 2011). Comparable distortions favoring sexual individuals are characteristic of segregating populations of other apomictic plants (Ozias-Akins and van Dijk, 2007).

The most common hypothesis to explain the low transmission rate of apomixis in segregating populations is the presence of a lethal allele linked to the apomixis locus acting at either the gametophytic or sporophytic level (Ortiz et al., 2013). Molecular analyses in *P. notatum*, *P. simplex*, and *P. procurrens* Quarin have shown strong repression of recombination around the ACL (Martínez et al., 2003; Pupilli et al., 2004; Stein et al., 2004; Hojsgaard et al., 2011). The presence of a chromosome rearrangement (i.e., paracentric inversion) could explain both the distorted segregation ratio

of apospory, via differential survival of meiocytes carrying the ACL, and the observed suppression of recombination near that locus in apomictic *P. notatum* (Stein et al., 2004). Recently, meiotic abnormalities were demonstrated to be associated with apospory in this species (Podio et al., 2012). Similarly, a translocation could be involved in the chromosome segment carrying the apomixis factors in *P. simplex* (Pupilli et al., 2004). Additional cytological studies in *P. guenoarum* would be necessary to investigate if a chromosome rearrangement is associated with the ACL in the species and whether it could explain the segregation proportions for reproductive mode here observed.

Alternatively, postmeiotic factors favoring the development of sexual embryos were invoked to explain the preferential transmission of sexuality in segregating populations of *Hieracium* spp. (Bicknell et al., 2000). In that direction, Polegri et al. (2010) proposed that in systems involving sexual and apomictic parents, the sexual genotype that receives the apomixis locus should regulate a network of genes acting downstream of the apomixis linked factors. If this sexual genotype is unable to regulate properly the interaction of these genes for example due to the genetic distance between the parents, as in interspecific crosses, it could ultimately result in apomictic lethal zygotes. Thus, zygotic lethality also may account for the low transmission rate of apomixis in *Paspalum* spp. and may be one of the reasons for segregation distortion observed between species of the Plicatula group. Experimental evidence supporting this hypothesis was detected in an interspecific cross between *P. simplex* and *P. procurrens* in which highly distorted segregation against apomictic progenies was observed (Hojsgaard et al., 2011).

Analysis of the apomixis expressivity in a random sample of 35 F_1 apomictic hybrids showed that all plants were highly or fully apomictic with few differences between individuals (Table 3). This outcome indicated that the transmission of the determinants of the trait lead to obligated or nearly obligated apomictic reproduction. At the same time, a low degree of sexuality in the 35 analyzed plants was observed. As a whole, our results indicate that in the F_1 population, segregation for the mode of the reproduction clearly favored the presence of sexual individuals, but, when expressed (in apomictic hybrids), apomixis showed a strong bias against the development of meiotic embryo sacs.

There is evidence that the degree of apomixis can be influenced by several factors external to the maternal plant, such us the pollen donor, photoperiod, and temperature (Koltunow, 1993). In *P. notatum*, the lowest expression of residual sexuality was coincident with the maximum flowering period (Rebozzio et al., 2011). *Paspalum cromyorrhizon* Trin., a close relative of *P. notatum*, showed variation in the degree of apomictic expression correlated with different stages of flowering (Quarin, 1986). Recently, it has been demonstrated that the light–dark

conditions affect alternative splicing of a subset of *Arabidopsis thaliana* (L.) Heynh. genes preferentially encoding proteins involved in RNA processing (Petrillo et al., 2014). Whether light conditions, as an external factor related to photoperiod at maximum and minimum flowering period, regulate alternative splicing of *Paspalum* genes potentially involved in apomictic or sexual pathways is still a matter of further investigation.

In addition, among these 35 F_1 hybrids, 15 showed capability to form B_{III} seeds, which means double fertilization of aposporous embryo sacs (apospory but not parthenogenesis). This result suggests that determinants of apomictic development, that is, apospory and parthenogenesis, may occasionally act in an uncoupled manner. A similar finding of B_{III} seed hybrid formation was reported for 10 natural populations belonging to four *Paspalum* species (Sartor et al., 2011). However, the only two clearly documented cases of recombination between apospory and parthenogenesis were described for *Poa pratensis* L. (Albertini et al., 2001) and *Cenchrus ciliaris* L. (Conner et al., 2013).

Apomixis is a heritable mode of reproduction thought to have evolved through a rearrangement of the developmental programs that constitute the normal sexual pathway (Grimanelli et al., 2001). However, no genes associated with dominant loci governing unreduced gamete formation and parthenogenesis have been linked to control components of natural apomixis (Rodriguez-Leal and Vielle-Calzada, 2012). Genes differentially expressed in space and time between apomictics and their sexual counterparts have been described in several species (reviewed in Pupilli and Barcaccia, 2012, Barcaccia and Albertini, 2013). Corral et al. (2013) recently suggested that the expression of a unique deregulated allele could trigger a series of events at the cellular level leading to asexual female gamete formation. These authors identified *APOLLO*, a gene encoding an exonuclease, whose transcripts are downregulated in premeiotic sexual ovules and upregulated in apomeiotic ovules at the same stage of development in *Boechera* spp. This gene is highly polymorphic and showed both "apoalleles," characterized by several apomixis-specific polymorphisms, and "sexalleles" (Corral et al., 2013). The expression of these apoalleles was strongly associated with apomixis. Apomictic plants were heterozygous showing at least one apoallele and one sexallele, and sexual genotypes were homozygous for sexalleles. However, there is no genetic evidence supporting that the sole transmission of apoalleles to the progeny is sufficient to confer the apomictic reproduction. Also, aposporous *Hypericum perforatum* L. has shown aposporic and sexual-specific alleles of the gene *HpARI7* (homologous to *Arabidopsis ARIADNE7*) at the tetraploid level. Aposporous plants presented a simplex genotype carrying one "apo" allele and three sex alleles, while sexual plants have a quadruplex genotype with four copies of the sex

allele. While aposporic and sexual alleles are coexpressed in the pistils at different developmental stages, the apomictic allele was shown to be specifically expressed in pistils of apomictic individuals (Schallau et al., 2010). These are just two examples, among many others, of candidate genes for apomixis genetic control.

Whether the products of genes preferentially expressed in apomictic plants are proteins that are not produced in sexual plants or proteins that normally function to initiate events in sexual reproduction but have become altered still remains unclear (Barcaccia and Albertini, 2013). In this regard, it should be considered that apomixis inheritance and expressivity appear to be more complex than expected. Molecular and cytogenetic analyses in several species suggest there is a more complex inheritance mechanism for apomixis, which could involve a system of polygenes, lack of recombination, *trans*-acting elements for gamete elimination, heterochromatic regions with abundance of transposable elements, or gene silencing through RNA (Pupilli and Barcaccia, 2012; Barcaccia and Albertini, 2013).

The tetraploid F_1 germplasm developed in this work offers the possibility of manipulating aposporous apomixis for fixing hybrids with desirable agronomic characteristics. Also, this type of population proved to be a good system for transferring the variability contained in natural apomictic races of *P. guenoarum* for breeding purposes. Fertility of most F_1 individuals in open pollination conditions is fundamental for basic genetic studies as well as for breeding purposes. The ability of these hybrids to produce seeds suggests that it will be possible to generate almost any array of crosses involving any sexual or apomictic plant. The agronomic evaluation of the highly apomictic hybrids could identify superior genotypes for forage improvement. And more importantly, sexual and apomictic hybrids generated are now available to be used as parents in future breeding programs.

Acknowledgments

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