RESEARCH

Breeding Tetraploid *Paspalum simplex*: Hybridization, Early Identification of Apomicts, and Impact of Apomixis on Hybrid Performance

E. A. Brugnoli, E. J. Martínez, S. C. Ferrari Usandizaga, A. L. Zilli, M. H. Urbani, and C. A. Acuña*

ABSTRACT

Apomictic grasses predominate among tropical forages, and specific breeding techniques are needed for their genetic improvement. The objectives of this study were (i) to generate hybrids by crossing tetraploid sexual and apomictic *Paspalum simplex* Morong genotypes, (ii) to develop a technique based on molecular markers for early identification of apomictic hybrids, and (iii) to determine the relationship between mode of reproduction and performance of hybrids. Crosses were made between two induced sexual and seven apomictic tetraploid plants. Identification of apomictic hybrids during seedling stage was performed using a marker linked to apomixis, and by flow cytometric seed analysis. Hybrids were evaluated under field conditions for plant diameter and height, initial growth, and seasonal regrowth during two consecutive growing seasons. Sexual tetraploid plants used as female parents behaved as allogamous, since 95% of the progeny had male-specific markers. The ratio between apomictic and sexual hybrids differed from 1:8.7 to 1:0.6 among crosses, with a mean of 1:2.4. There was a 96% coincidence between the use of the apomixis-linked marker and flow cytometric seed analysis. A technique based on the use of early DNA isolation and the amplification of a molecular marker linked to apomixis was developed. We found no overall difference between apomictic and sexual hybrids for the evaluated traits, except for fall regrowth in the first year, for which apomictic hybrids were superior. Generation of large tetraploid hybrid progenies is possible in *P. simplex*. Segregation for mode of reproduction depends on parents involved. Agronomic performance is not related to reproductive mode among tetraploid hybrids.

E.A. Brugnoli, E.J. Martínez, A.L. Zilli, M.H. Urbani, and C.A. Acuña, Instituto de Botánica del Nordeste, Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Agrarias, Univ. Nacional del Nordeste, Sargento Cabral 2131, Corrientes, Argentina; S.C. Ferrari Usandizaga, Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Corrientes, Ruta Nacional 12, Km 1008, Corrientes, Argentina. Received 28 Dec. 2018. Accepted 21 Mar. 2019. *Corresponding author (cacuna@agr.unne.edu.ar). Assigned to Associate Editor Bradley Bushman.

Abbreviations: ISSR, inter simple sequence repeat; PCR, polymerase chain reaction; SCAR, sequence characterized amplified region; UV, ultraviolet.

Apomixis, asexual reproduction through seeds, is present in ~300 species of 33 families (Carman, 1997). Apomictic reproduction is common in the warm-season (C_4) grasses (Vogel and Burson, 2004) and plays a key role in the colonization of new environments and in defining the allocation of plant diversity (Brugnoli et al., 2013, 2014).

Apomixis offers the opportunity of fixing heterozygous genotypes over seed-propagated generations (Hanna and Bashaw, 1987). Although transfer of apomixis to the most important food crops has not been successful, the trait can be exploited in breeding those crops where it occurs naturally. The common method used for cultivar development in apomictic species has been the selection of ecotypes with desirable characteristics in target environments (Vogel and Burson, 2004). Most current cultivars of apomictic forage grasses have been selected from the available germplasm. One example is the cultivar Marandu of *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf, which is cultivated in a vast tropical and subtropical area around the world (Jank et al., 2014).

It is also possible to release the diversity present in wild genotypes, which are polyploid, by crossing them with sexual germplasm that shares the same ploidy level. These sexual plants,

Published in Crop Sci. 59:1617–1624 (2019).
doi: 10.2135/cropsci2018.12.0771

which are the key for crossing at the polyploid level, are mainly obtained from sexual diploid germplasm by chromosome duplication (Miles, 2007). Hybridization allows the generation of populations segregating for agronomic traits and mode of reproduction. This method has been used in apomictic grass species of several genera (e.g., *Brachiaria*, *Panicum*, *Cenchrus*, and *Paspalum*; Vogel and Burson, 2004).

One of the limiting factors for improving apomictic species is the identification of apomictic hybrids within segregating progeny (Savidan et al., 2001). Most available methods rely on reproductive tissues or observation of progeny. Embryo sac observation is the most popular method among plant breeders (Weiler et al., 2017; Zilli et al., 2018), since it is inexpensive and reliable. Flow cytometric seed analysis is also effectively used for some species (Novo et al., 2017). Field progeny tests are based on observing the homogeneity within the progeny (Burton, 1992). This can be based on gross morphology or by analyzing the molecular fingerprinting with molecular markers (Acuña et al., 2005).

Where reliable apomixis-linked molecular markers are available, an early classification for mode of reproduction can be accomplished using seedling tissue, improving the efficiency of the breeding process. Only those seedlings identified as apomictic need be evaluated under field conditions. Although there are several molecular markers linked to apomixis in warm-season grasses, only a few have been determined to be reliable for breeding purposes. Two random amplified polymorphic DNA (RAPD) markers, previously determined to be linked to apomixis in *Panicum maximum* Jacq., were used to identify apomictic hybrids in two F_1 populations with an efficiency varying from 77.3 to 90% (Bluma Marques et al., 2014). Kumar et al. (2017) tested the efficiency of four sequence characterized amplified region (SCAR) markers linked to apomixis, and one SCAR linked to sexuality in *Cenchrus ciliaris* L., on a segregating F_2 population; they found that these markers were able to distinguish apomictic plants from sexual ones but failed to discriminate between facultative apomictic and sexual plants.

The progeny resulting from crosses between compatible sexual and apomictic genotypes is usually highly diverse for agronomic traits (Acuña et al., 2011; Zilli et al., 2015; Weiler et al., 2018). The primary advantage of crossing sexual and apomictic strains is that the hybrid progenies segregate for mode of reproduction and traits of interest are fixed over generations (Hanna, 1995). However, little is known about the effect of apomixis on agronomic traits. Apomixis has been related to the accumulation of a high mutation load (Hojsgaard et al., 2015). Most mutations are expected to be deleterious. For example, the apo-locus in *Paspalum notatum* Flüggé appears to be linked to a lethal allele responsible for the distorted segregation ratios (Martínez et al., 2001). However, fully sexual, polyploid plants have not been found in nature for the most important apomictic forage species (Vogel and Burson, 2004). This fact has been related to the colonization capacity of apomicts (Brugnoli et al., 2014). The evaluation of progeny segregating for apomixis and a series of agronomic traits may provide information about the relation between mode of reproduction and agronomic performance.

Paspalum simplex Morong is a Poaceae native to South America (Urbani et al., 2002). The species has potential as a forage crop in semiarid regions (Brugnoli et al., 2014). Further, *P. simplex* is considered a model species for genetic studies because it forms an agamic complex with several cytotypes and different modes of reproduction. Tetraploid apomictic populations are the most common in nature and are present throughout the entire range of the species (Caponio and Quarin, 1987; Urbani et al., 2002). The diploid cytotype, which reproduces sexually (Espinoza and Quarin, 1997), has been found only in the north of Argentina (Urbani et al., 2002; Brugnoli et al., 2013). Previous studies have shown that is possible to obtain apomictic hybrids in *P. simplex* (Pupilli et al., 1997, Cáceres et al., 1999). A SCAR marker completely linked to apospory in tetraploid *P. simplex* is available (Calderini et al., 2006). This species represents an interesting case for evaluating the relationship between mode of reproduction and agronomic traits.

The objectives of the present work were (i) to generate tetraploid hybrids by crossing induced tetraploid sexual and apomictic *Paspalum simplex* genotypes, (ii) to develop a technique based on molecular markers for early identification of apomictic hybrids, and (iii) to determine the relationship between mode of reproduction and agronomic performance.

MATERIALS AND METHODS

Plant Material

Crosses were made between tetraploid sexual and apomictic genotypes of *P. simplex* in February and March 2010. Two sexual tetraploid clones C_{1-2} (Hojsgaard et al., 2008) and C_1B_2 (Hojsgaard et al., 2011) were used as female parents. These plants derived from self-pollination of a colchicine-induced autotetraploid plant obtained in the Istituto di Ricerche sul Miglioramento Genetico delle Piante Foraggere del Consiglio Nazionale delle Ricerche, Perugia, Italy (Cáceres et al., 1999). The apomictic ecotypes used as male parents were collected from different locations of Argentina—Reconquista (29°08′ S, 59°39′ W), Mercedes (29°10′ S, 58°04′ W), Santa Ana (27°27′ S, 58°39′ W), Piedras Blancas (31°11′ S, 59°57′ W), Villa Ángela (27°35′ S, 60°44′ W), Castelli (25°56′ S, 60°36′ W)—and one location from southern Brazil—Porto Murtinho (21°42′ S, 57°51′ W) (Table 1). Crosses were made at the Instituto de Botánica del Nordeste, Corrientes, Argentina. Plants used as female parents were grown in pots under a greenhouse, whereas

Table 1. The number of pollinated florets and seed set from crosses made between sexual and apomictic tetraploid genotypes of *Paspalum simplex*.

Female sexual parents	Male apomictic parents	Identification family	Pollinated florets	Seed set
			no.	%
C_{1-2}	Mercedes	A	663	40.1
C_{1-2}	Porto Murtinho	B	1,436	26.1
C_{1-2}	Piedras Blancas	C	1,728	24.2
C_{1-2}	Reconquista	D	1,914	18.6
C_{1-2}	Villa Ángela	E	1,661	14.2
C_1B_2	Castelli	F	1,877	51.4
C_1B_2	Porto Murtinho	G	813	47.3
C_1B_2	Santa Ana	H	2,140	32.6
Total			12,232	

male parents were grown in the experimental field of Facultad de Ciencias Agrarias (27°28′ S, 58°47′ W). The procedure consisted of collecting inflorescences of male parents a day before anthesis. These were kept in containers with water in the greenhouse until pollination. Female inflorescences were not emasculated. Pollen from each male parent was collected in glassine bags and deposited on the inflorescences of the female parent at anthesis (around 8:00 AM). After pollination, inflorescences were isolated with glassine bags for 15 to 20 d until seed maturation. Seeds were stored for ∼6 mo, until the start of the next growing season. Approximately 150 seeds from each family were sown in trays with sterile soil in a greenhouse in September 2010. The seedlings were transplanted to 500-mL pots in October 2010. About 30 plants at random were kept from each of the parental combinations.

Efficiency of the Crossing Technique

A random sample of five plants of the progeny of each family was evaluated to determine the efficiency of the crossing technique. Hybrid origin of the progenies was evaluated by DNA fingerprinting using inter simple sequence repeat (ISSR) markers. Genomic DNA was extracted using the protocol described by Brugnoli et al. (2014). Approximately 50 mg of young leaves was used for DNA extraction. Leaves were macerated with the help of a plastic fuse drill and 700 μL of extraction buffer cetyl trimethylammonium bromide (CTAB) 2% (100 mM Tris-HCl, pH 7.5; 50 mM ethylenediaminetetraacetic acid [EDTA], pH 8; 700 mM NaCl; and 140 mM β-mercaptoethanol) in a tube of 1.5 mL. Samples were incubated at 65°C for 30 min, and then 500 μL of chloroform was added and the mixture was stirred for 5 min and centrifuged for 10 min. The aqueous phase was recovered and transferred to another tube. The nucleic acids were precipitated with 500 μL of cold 2-propanol. Tubes were kept in a freezer at −20°C for ∼30 min, and then were centrifuged at 4°C for 20 min. The supernatant was discarded, and the obtained pellet was washed with a 70% ethanol solution containing 0.2 M sodium acetate, and centrifuged again for 10 min. After centrifugation, the supernatant was discarded and the pellet was suspended in 25 μL of sterile TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA, pH 8) and kept in a refrigerator. Genomic DNA was quantified by visual comparison with a known concentration DNA pattern, by electrophoresis in agarose 1% (w/v) gels in 1× TAE buffer (40 mM Tris-HCl; 5 mM sodium acetate; 0.77 mM EDTA, pH 8.0) at 40 V for 2 h. The DNA was visualized under ultraviolet (UV) light and photographed with GelDoc-It Imaging System (UVP), after staining with ethidium bromide (10 μg mL^{-1}). Each DNA samples was adjusted to 20 ng μL^{-1} for their use in polymerase chain reaction (PCR). We identified ISSR polymorphisms between female and male parents. Two or more specific bands of male parents were considered to confirm hybrid origin. The ISSR amplification was performed using a technique described by Brugnoli et al. (2014).

Identification of Apomictic Hybrids

Identification of apomictic hybrids was performed using seedlings or young plants (four leaves). Genomic DNA was isolated using the technique previously described. A *Paspalum simplex* apomixis-specific SCAR (Labombarda et al., 2002; Calderini et al., 2011) was screened on 30 hybrids of each family. The PCR reactions were performed in a 25-μL final volume containing 1 μL of template DNA (20 ng), 2.5 μL of reaction buffer 10× (includes 50 mM of $MgCl_2$), 2 μL of forward primer (0.4 μM), 2 μL of reverse primer (0.4 μM), 1.25 μL of deoxynucleotides (100 μM), 0.2 μL of Taq DNA polymerase Promega (1 U), and H_2O to complete 25 μL. The DNA amplifications were performed in a thermal cycler (Applied Biosystems Gene AMP PCR system 2400) with the following thermal cycle: initial denaturing at 93°C for 1 min; 30 cycles of 93°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were separated by electrophoresis in 2% (w/v) agarose gels in 1× TAE buffer at 70 V for 2 h and stained with ethidium bromide (10 μg mL^{-1}). Molecular profiles were visualized under UV light, photographed, and stored for further analysis with GelDoc-It Imaging System.

Sequenced characterized amplified region products were scored for the presence (1) or absence (0) of a 650-bp, apomixis-specific marker. Hybrids that amplified this marker were classified as apomictic, whereas those that did not were considered as sexual.

Flow Cytometric Seed Screen

Seeds were collected from a sample of 84 hybrids (10%) at random from three parental combinations during summer 2012. Flow cytometric seed screen (Matzk et al., 2000) was used to determine the mode of reproduction of each hybrid and evaluate the efficiency of using the SCAR for the same purpose. Between 10 and 50 seeds from each hybrid were analyzed. Nuclei were isolated by chopping two seeds because this form is clearer evidence for facultative apomixis than the bulked analysis, with a razor blade in 0.5 mL of nuclei isolation buffer (Partec P kit CyStain UV). Samples were incubated for 2 min and then filtered through a 30-μm nylon mesh directly into the sample tube, to which 1.5 mL of fluorescent stain 4α,6-diamidino-2-phenylindole (DAPI) staining buffer (Partec P kit CyStain UV) was added. The mixture was incubated for 5 min at room temperature and analyzed with a Partec PA II (Ploidy Analyzer II) flow cytometer with the detector operating at 355 nm. At least 3000 nuclei were counted for each sample (two seeds). Data were analyzed using PA-II Partec FloMax software. The mean values of DNA content for embryo and endosperm were established to infer the sexual or apomictic origin of each seed.

The relative embryo/endosperm DNA content was expected to be 4*x*/6*x* in a sexually formed seed, resulting from a 4*x* (*n* + *n*) embryo and a 6*x* (*n* + *n* + *n*) endosperm. In contrast, a relative embryo/endosperm DNA content of 4*x*/10*x* was expected in an apomictically formed seed, because the embryo was formed by parthenogenesis of an unreduced egg cell (2*n* + 0), whereas the endosperm arises from pseudogamy that involves the central cell (carrying two unreduced polar nuclei) fertilized by one reduced sperm cell of the pollen tube (2*n* + 2*n* + *n*). These embryo/endosperm ratios of DNA content in sexual and apomictic plants were previously determined for *P. simplex* by Galdeano et al. (2016). Figure 1 illustrates an example of the determination of reproductive mode based on flow cytometry.

Pearson correlation coefficient analysis between the results obtained with the SCAR and flow cytometry were performed using InfoGen software (Balzarini and Di Rienzo, 2013).

Comparison between Sexual and Apomictic Hybrids for Morphological and Agronomic Traits

Thirty hybrids resulting from each cross were used for comparisons between sexual and apomictic hybrids. These 240 hybrids were transplanted to the field on 1-m centers in a completely randomized design. The number of replications for sexual and apomictic was dependent on segregation for mode of reproduction. The experiment was planted in Corrientes, Argentina (27°40′ S, 58°45′ W) in December 2010. The soil type was classified as Argiudoll, with 4.5 pH, 3.4% organic matter, and 4.7 mg P kg^{-1}.

The morphological traits evaluated were plant diameter and height. Plant diameter was determined using the average between the longest and shortest diameter of a given plant, whereas plant height was measured from the soil surface to the top of the canopy.

Seasonal growth was evaluated during two production cycles (2011 and 2012). Growth was estimated using a 1-to-5 visual scale, where 1 represents plants exhibiting the least growth, and 5 represents plants with the greatest growth. Initial growth was estimated in March 2011. Plants were defoliated to leave an approximately 10-cm stubble height in April 2011. Fall regrowth was estimated 30 d after defoliation. Winter regrowth was evaluated on 19 Aug. 2011, using a visual scale from 1 to 5. Fall and winter regrowth was evaluated again during April 2012 and August 2012.

Comparisons between sexual and apomictic hybrids for each trait were made using ANOVA and the Tukey's test. Reproductive mode comparisons were made within each family and over all families.

RESULTS

Generation of F_1 Families

Eight families were obtained from crosses between two sexual female parents and seven apomictic male parents (Table 1). Average seed set was 32%, ranging from 14 to 51% among crosses.

The reliability of the crossing technique was evaluated using four ISSR primers on five plants per family (40 plants total). Only two plants (5%) did not amplify the male-parent-specific ISSR markers and therefore were considered the result of self-pollination. Thus, the efficiency of the crossing method without previous emasculation of the female parent was 95%.

Segregation for Mode of Reproduction

Two hundred and thirty-two hybrids were classified for mode of reproduction by means of an apomixis-specific SCAR marker of *P. simplex*. A total of 68 hybrids amplified this marker and were identified as apomictic. In addition, the proportion of apomictic hybrids differed among families (Table 2). Two families (A and H) had

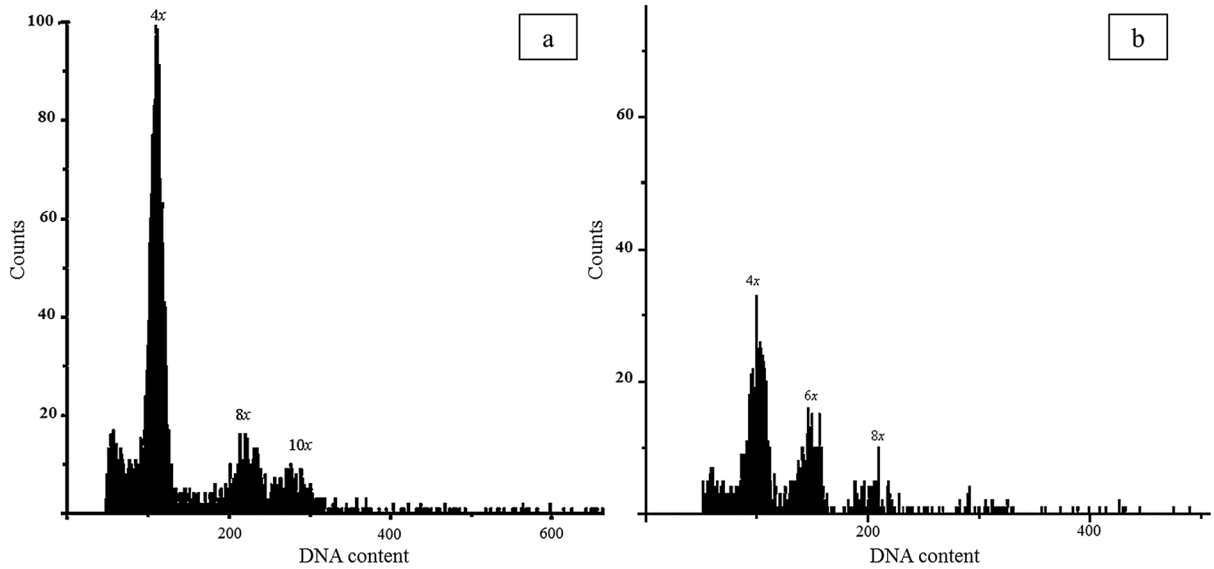

Fig. 1. DNA content histogram generated by flow cytometry. (a) Two apomictic seeds of *Paspalum simplex*. The highest peak corresponds to the embryo (4×), the intermediate is nuclei at the G2 stage of the cell cycle (8×), and the lowest is endosperm cells (10×). (b) Two sexual seeds. The largest peak corresponds to the embryo (4×), intermediate is the endosperm (6×), and lowest is nuclei at the G2 stage of the cell cycle (8×).

Table 2. Reproductive classification by mean a specific-apomixis sequence characterized amplified region (SCAR) molecular marker of *Paspalum simplex*. Number of hybrids, number of apomictic and sexual hybrids, and the ratio between apomictic (Apo) and sexual (Sex) plants in eight segregating families of *P. simplex* are shown.

Family	Hybrids analyzed	Apomictic hybrids	Sexual hybrids	Apo/Sex ration
	no.			
A	28	18	10	1:0.6
B	29	3	26	1:8.7
C	30	6	24	1:4.0
D	30	4	26	1:6.5
E	29	4	25	1:6.3
F	30	8	22	1:2.8
G	26	6	20	1:3.3
H	30	19	11	1:0.6
Overall	232	68	164	1:2.4

a greater number of apomictic hybrids, exceeding the sexual hybrids in both cases. Over all families, the average segregation ratio was 1:2.4 (apomictic/sexual).

Flow cytometry was used for 84 hybrids from three families. The classification for mode of reproduction by flow cytometry and SCAR marker matched in 96% of the cases. A difference of 4% (three hybrids) was observed in two hybrids of family A and one hybrid of family G. These three hybrids, classified as apomictic through the SCAR marker, were considered sexual by flow cytometry. The two techniques matched perfectly for all the hybrids of family C (Table 3). The Pearson correlation coefficient between the two techniques was highly significant (r = 0.92, $p \leq 0.0001$).

Comparison between Sexual and Apomictic Hybrids for Morphological and Agronomic Traits

Variation among and within families of *Paspalum simplex* was observed for all evaluated traits. Comparisons between hybrids with different modes of reproduction were made for each family. Sexual and apomictic hybrids did not differ for plant diameter, height, initial growth, and spring regrowth. In family G, apomictic hybrids were superior for fall regrowth in the first year. Differences for winter regrowth in the first year were observed for three families. Apomictic hybrids of family C were inferior to sexual ones, whereas the apomictic hybrids of families A and D were superior to the sexual ones. Sexual and apomictic hybrids did not differ for spring regrowth. Reproductive modes did not differ for fall and winter regrowth in the second year except for two families. The apomictic hybrids of families A and E were superior to the sexual ones for fall regrowth (Table 4).

When reproductive modes were compared across families, no differences were observed between sexual and apomictic hybrids for plant diameter, plant height, initial growth, or winter and spring regrowth in either year of evaluation. Reproductive modes differed for fall regrowth in the first evaluated year, with apomictic hybrids being superior (Table 4). These differences for fall regrowth were not observed in the second year.

DISCUSSION

Modern breeding programs for apomictic species are dependent on efficient generation of hybrids, early and simple identification of apomicts that enter in a forage evaluation process, and a high level of heterosis for traits of agronomic interest. In this research, we evaluated the efficiency of sexual × apomictic crosses in tetraploid *P. simplex*. The two sexual clones were developed in Italy (Cáceres et al., 1999), and the results of this research indicate that they only produce seeds by a sexual process. This is critical, since facultative apomictic plants may result from the chromosome duplication process in *Paspalum* (Quarin and Hanna, 1980; Quarin et al., 2001; Acuña et al., 2007). Additionally, these two clones are largely self-incompatible: 95% of the progeny generated by crosses made without emasculation were hybrids. This finding is also important because sexual tetraploid hybrids can be highly self-fertile (Acuña et al., 2009). Apomictic accessions from diverse origins were used and all generated large numbers of hybrids.

Early identification of apomicts is expected to speed up the breeding process of apomictic species. The use of specific markers linked to apomixis is an attractive option to achieve this goal. For the success of this methodology, it is necessary to have markers linked to the apospory locus. These markers must be reliable and preferably easy to use and inexpensive. In this work, we demonstrated that the SCAR marker developed by Calderini et al. (2006) can be used for an efficient identification of apomictic hybrids at the seedling stage in *P. simplex*. Classification by molecular

Table 3. Apospory identification of tetraploid hybrids of *Paspalum simplex* analyzed by flow cytometry, and the degree of coincidence between the molecular and cytometric methods used.

Family	Hybrids analyzed	Apomictic hybrids	Sexual hybrids	Apo/Sex ration	Degree of coincidence between techniques
	no.				%
A	28	16	12	1:0.8	93
C	30	6	24	1:4.0	100
G	26	5	21	1:4.2	96
Total	84	27	57	1:2.1	96

Table 4. Means for morphological and agronomical traits recorded in two evaluated years for apomictic (Apo) and sexual (Sex) hybrids of families of *Paspalum simplex*.

					First year†						Second year†					
	Diameter		Height		Initial growth		Fall regrowth		Winter regrowth		Spring regrowth		Fall regrowth		Winter regrowth	
Family	Apo	Sex	Apo	Sex	Apo	Sex	Apo	Sex	Apo	Sex	Apo	Sex	Apo	Sex	Apo	Sex
	———— cm ————															
A	17.3	16.7	99.1	100.2	3.1	3.1	3.2	2.9	2.8*	2.2	3.0	2.8	3.1*	2.6	1.8	1.7
B	21.1	20.2	116.0	120.7	3.3	3.6	4.0	3.6	3.2	3.6	3.5	3.4	4.3	3.8	2.8	2.8
C	17.1	18.9	96.1	103.1	3.1	3.2	3.1	3.4	2.1**	2.7	2.8	2.9	3.3	3.3	2.0	1.9
D	19.0	18.1	110.6	116.8	3.6	3.3	3.8	3.4	3.4*	2.8	3.1	3.2	3.8	3.4	3.0	2.7
E	17.3	17.9	107.6	113.6	3.0	3.0	3.6	3.4	3.5	3.3	3.4	3.4	4.3*	3.5	2.5	2.5
F	16.1	16.2	104.7	99.0	3.1	2.9	3.1	2.9	3.0	3.0	2.9	2.5	3.1	3.2	2.7	2.4
G	19.3	17.9	117.7	107.0	3.7	3.6	4.0*	3.3	4.2	4.2	3.8	3.3	4.3	4.0	3.9	3.5
H	18.3	16.5	118.3	117.3	3.8	3.5	3.8	3.7	3.6	3.4	3.6	3.4	3.7	3.6	2.8	2.9
Overall	17.8	18.0	108.9	109.5	3.4	3.3	3.5***	3.3	3.2	3.2	3.3	3.1	3.6	3.5	2.6	2.6

* Significant difference between sexual and apomictic at the 0.05 probability level.

** Significant difference between sexual and apomictic at the 0.01 probability level.

*** Significant difference between sexual and apomictic at the 0.001 probability level

† Estimated using a visual scale from 1 to 5, where 1 is the lowest and 5 is the highest.

markers or flow cytometry was highly consistent. Two hybrids classified as apomictic by the SCAR marker were identified as sexual by flow cytometry. This difference may be due to an expression of apospory in these hybrids, which was not detected by flow cytometry; therefore, a greater number of seeds should be analyzed.

Several attempts have been made to develop molecular markers that can be used to discriminate between sexual and apomictic genotypes resulting from crosses in other genera, (e.g., *Brachiaria*, *Poa*, *Cenchrus*, and *Panicum*; Pessino et al., 1997; 1998; Albertini et al., 2001; Dwivedi et al., 2007; Zorzatto et al., 2010; Bluma Marques et al., 2014; Worthington et al., 2016; Kumar et al., 2017). However, few reports of the use of these markers are available. Worthington et al. (2016) reported that a single SCAR marker (N14) is regularly used in the *Brachiaria* breeding program of CIAT. The authors note that this marker is reliable only when *B. decumbens* Stapf cultivar Basilisk is used as the male parent. The marker used in the present study for *P. simplex* was tested using nine apomictic parents collected from contrasting locations throughout the region of natural distribution for the species (Brugnoli et al., 2014), indicating a high efficiency independently of the male genotype used.

We observed different segregation for mode of reproduction among the eight families generated in the present study. More sexual than apomictic hybrids were observed, with a mean segregation ratio of 1:2.4 (apomictic/sexual). This segregation pattern differs from that expected for a cross between a nulliplex (aaaa) female parent and a simplex (Aaaa) male parent for the "apo locus," the generally accepted model in warm-season grasses (Miles, 2007). These results are in agreement with observations for several other *Paspalum* species, as reviewed by Ortiz et al. (2013). The greater proportion of apomicts we observed in progeny from two crosses has not previously been reported for the genus. Further research is needed to clarify the source of the observed differences among families.

For most agronomic traits, apomictic and sexual hybrids did not differ. The results show that mode of reproduction does not necessarily improve or decrease agronomic performance. Although apomixis has been linked to the accumulation of a high mutation load (Hojsgaard et al., 2015), our results suggest that apomixis, per se, does not have a negative effect on agronomic traits. Sexual hybrids do not have a disadvantage in agronomic performance compare with the apomictic plants, indicating that other factors are responsible for the failure to find fully sexual tetraploid plants in nature. The success of apomictic tetraploid plants in nature may be related to the ability to colonize new environments by asexual means, as has previously been proposed (Brugnoli et al., 2014).

CONCLUSIONS

A large number of hybrids can be generated in *P. simplex* using crosses between induced sexual and apomictic tetraploid plants. Sexuality and self-incompatibility of the available synthetic germplasm assure the generation of hybrids. Segregation for apomixis in *P. simplex* was highly variable and depended on the combination of parents used, although the mean is not different from other *Paspalum* species. A technique based on the use of early DNA isolation and the amplification of a molecular marker linked to apomixis was developed for a quick and simple identification of apomictic hybrids in *P. simplex*. The results also indicate that mode of reproduction does not affect agronomic performance of tetraploid hybrids at the individual plant level.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgments

The authors would like to thank Dr. John W. Miles for reviewing this manuscript and Florencia Galdeano for technical assistance. This research was carried out with contributions from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina, PICT no. 2008-00264, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) PIP 11420100100031, Secretaría General de Ciencia y Técnica (Universidad Nacional del Nordeste) PI A001-2009 and PI A005-2010, and Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste.

References

Acuña, C.A., A.R. Blount, K.H. Quesenberry, W.W. Hanna, and K.E. Kenworthy. 2007. Reproductive characterization of bahiagrass germplasm. Crop Sci. 47:1711–1717. doi:10.2135/cropsci2006.08.0544

Acuña, C.A., A.R. Blount, K.H. Quesenberry, K.E. Kenworthy, and W.W. Hanna. 2009. Bahiagrass tetraploid germplasm: Reproductive and agronomic characterization of segregating progeny. Crop Sci. 49:581–588. doi:10.2135/cropsci2008.07.0402

Acuña, C.A., A.R. Blount, K.H. Quesenberry, K.E. Kenworthy, and W.W. Hanna. 2011. Tetraploid bahiagrass hybrids: Breeding technique, genetic variability and proportion of heterotic hybrids. Euphytica 179:227–235. doi:10.1007/s10681-010-0276-y

Acuña, C.A., E.J. Martínez, and C.L. Quarin. 2005. Sexual diploid and apomictic tetraploid races in *Thrasya petrosa* (Gramineae). Aust. J. Bot. 53:479–484. doi:10.1071/BT04190

Albertini, E., G. Barcaccia, A. Porceddu, S. Sorbolini, and M. Falcinelli. 2001. Mode of reproduction is detected by Parth1 and Sex1 SCAR markers in a wide range of facultative apomictic Kentucky bluegrass varieties. Mol. Breed. 7:293–300. doi:10.1023/A:1011673112747

Balzarini, M.G., and J.A. Di Rienzo. 2013. InfoGen versión 2013. Univ. Nac. Córdoba, Córdoba, Argentina. http://www.infogen.com.ar (accessed 17 Nov. 2018).

Bluma Marques, A.C., L. Chiari, D.C. Agnes, L. Jank, and M.S. Pagliarini. 2014. Molecular markers linked to apomixis in *Panicum máximum* Jacq. Afr. J. Agric. Res. 13:2198–2202.

Brugnoli, E.A., M.H. Urbani, C.L. Quarin, E.J. Martínez, and C.A. Acuña. 2013. Diversity in diploid, tetraploid and mixed diploid-tetraploid populations of *Paspalum simplex* Morong. Crop Sci. 53:1509–1516. doi:10.2135/cropsci2012.08.0497

Brugnoli, E.A., M.H. Urbani, C.L. Quarin, A.L. Zilli, E.J. Martínez, and C.A. Acuña. 2014. Diversity in apomictic populations of *Paspalum simplex* Morong. Crop Sci. 54:1656–1664. doi:10.2135/cropsci2013.11.0780

Burton, G.W. 1992. Manipulating apomixis in *Paspalum*. In: J.H. Elgin and J.P. Miksche, editors, Proceedings of the Apomixis Workshop, Atlanta, GA. 11–12 Feb. 1992. Res. Bull. 104. USDA-ARS, Washington, DC. p. 16–19.

Cáceres, M.E., F. Pupilli, C.L. Quarin, and S. Arcioni. 1999. Feulgen-DNA densitometry of embryo sacs permits discrimination between sexual and apomictic plants in *Paspalum simplex*. Euphytica 110:161–167. doi:10.1023/A:1003772623703

Calderini, O., S.B. Chang, H. de Jong, A. Busti, F. Paolocci, S. Arcioni, et al. 2006. Molecular cytogenetics and DNA sequence analysis of an apomixis-linked BAC in *Paspalum simplex* reveal a non pericentromere location and partial microcolinearity with rice. Theor. Appl. Genet. 112:1179–1191. doi:10.1007/s00122-006-0220-7

Calderini, O., I. Donnison, L. Polegri, F. Panara, A. Thomas, S. Arcioni, and F. Pupilli. 2011. Partial isolation of the genomic region linked with apomixis in *Paspalum simplex*. Mol. Breed. 28:265–276. doi:10.1007/s11032-010-9480-7

Caponio, I., and C.L. Quarin. 1987. El sistema genético de *Paspalum simplex* y de un híbrido interespecífico con *P. dilatatum*. Kurtziana 19:35–45.

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

Dwivedi, K.K., S.R. Bhat, V. Bhat, B.V. Bhat, and M.G. Gupta. 2007. Identification of a SCAR marker linked to apomixis in buffelgrass (*Cenchrus ciliaris* L.). Plant Sci. 172:788–795. doi:10.1016/j.plantsci.2006.12.006

Espinoza, F., and C.L. Quarin. 1997. Cytoembryology of *Paspalum chaseanum* and sexual diploid biotypes of two apomictic *Paspalum* species. Aust. J. Bot. 45:871–877. doi:10.1071/BT96055

Galdeano, F., M.H. Urbani, M.E. Sartor, A.I. Honfi, F. Espinoza, and C.L. Quarin. 2016. Relative DNA content in diploid, polyploid, and multiploid species of *Paspalum* (Poaceae) with relation to reproductive mode and taxonomy. J. Plant Res. 129:697–710. doi:10.1007/s10265-016-0813-4

Hanna, W.W. 1995. Use of apomixis in cultivar development. Adv. Agron. 54:330–350.

Hanna, W.W., and E.C. Bashaw. 1987. Apomixis: Its identification and use in plant breeding. Crop Sci. 27:1136–1139. doi:10.2135/cropsci1987.0011183X002700060010x

Hojsgaard, D., E. Schegg, J.F.M. Valls, E.J. Martínez, and C.L. Quarin. 2008. Sexuality, apomixis, ploidy levels, and genomic relationships among four *Paspalum* species of the subgenus *Anachyris* (Poaceae). Flora 203:535–547. doi:10.1016/j.flora.2007.09.005

Hojsgaard, D.H., E.J. Martínez, C.A. Acuña, C.L. Quarin, and F. Pupilli. 2011. A molecular map of the apomixis-control locus in *Paspalum procurrens* and its comparative analysis with other species of *Paspalum*. Theor. Appl. Genet. 123:959–971. doi:10.1007/s00122-011-1639-z

Hojsgaard, D., M. Pellino, T.F. Sharbel, and E. Hörandl. 2015. Resolving genome evolution patterns in asexual plants. In: E. Hörandl and M.S. Appelhans, editors, Next generation sequencing in plant systematics. Vol. 158. Gantner, Ruggell, Liechtenstein. p. 119–153.

Jank, L., S.C. Barrios, C.B. do Valle, R.M. Simeão, and G.F. Alves. 2014. The value of improved pastures to Brazilian beef production. Crop Pasture Sci. 65:1132–1137. doi:10.1071/CP13319

Kumar, S., S. Saxena, and M.G. Gupta. 2017. Marker-assisted screening of breeding populations of an apomictic grass *Cenchrus ciliaris* L. segregating for the mode of reproduction. Crop Breed. Appl. Biotechnol. 17:10–17. doi:10.1590/1984-70332017v17n1a2

Labombarda, P., A. Busti, M.E. Cáceres, F. Pupilli, and S. Arcioni. 2002. An AFLP marker tightly linked to apomixis reveals hemizygosity in a portion of the apomixis-controlling locus in *Paspalum simplex*. Genome 45:513–519. doi:10.1139/g02-014

Martínez, E.J., M.H. Urbani, C.L. Quarin, and J.P.A. Ortiz. 2001. Inheritance of apospory in bahiagrass, *Paspalum notatum*. Hereditas 135:19–25. doi:10.1111/j.1601-5223.2001.00019.x

Matzk, F., A. Meister, and I. Schubert. 2000. An efficient screen for the reproductive pathways using mature seeds of monocots and dicots. Plant J. 21:97–108. doi:10.1046/j.1365-313x.2000.00647.x

Miles, J.W. 2007. Apomixis for cultivar development in tropical forage grasses. Crop Sci. 47:S-238–S-249. doi:10.2135/cropsci2007.04.0016IPBS

Novo, P.E., C.A. Acuña, C.L. Quarin, M.H. Urbani, F. Marcón, and F. Espinoza. 2017. Hybridization and heterosis in the Plicatula group of *Paspalum*. Euphytica 213:198. doi:10.1007/s10681-017-1983-4

Ortiz, J.P.A., C.L. Quarin, S.C. Pessino, C.A. Acuña, E.J. Martínez, F. Espinoza, et al. 2013. Harnessing apomictic reproduction in grasses: What we have learned from *Paspalum*. Ann. Bot. 112:767–787. doi:10.1093/aob/mct152

Pessino, S.C., C. Evans, J.P.A. Ortiz, I. Armstead, C.B. Do Valle, and M.D. Hayward. 1998. A genetic map of the apospory-region in *Brachiaria* hybrids: Identification of two markers closely associated with the trait. Hereditas 128:153–158. doi:10.1111/j.1601-5223.1998.00153.x

Pessino, S.C., J.P.A. Ortiz, O. Leblanc, C.B. do Valle, and M.D. Hayward. 1997. Identification of a maize linkage group related to apomixis in *Brachiaria*. Theor. Appl. Genet. 94:439–444. doi:10.1007/s001220050434

Pupilli, F., M.E. Cáceres, C.L. Quarin, and S. Arcioni. 1997. Segregation analysis of RFLP markers reveals a tetrasomic inheritance in apomictic *Paspalum simplex*. Genome 40:822–828. doi:10.1139/g97-806

Quarin, C.L., F. Espinoza, E.J. Martínez, S.C. Pessino, and O.A. Bovo. 2001. A rise of ploidy level induces the expression of apomixis in *Paspalum notatum*. Sex. Plant Reprod. 13:243–249. doi:10.1007/s004970100070

Quarin, C.L., and W.W. Hanna. 1980. Effect of three ploidy levels on meiosis and mode of reproduction in *Paspalum hexastachyum*. Crop Sci. 20:69–75. doi:10.2135/cropsci1980.0011183X002000010016x

Savidan Y.H., J.G. Carman, and T. Dresselhaus. 2001. The flowering of apomixis: From mechanisms to genetic engineering. ClMMYT, Inst. Rech. Dév., European Commission, Mexico City.

Urbani, M.H., C.L. Quarin, F. Espinoza, M.I.O. Penteado, and I.F. Rodrigues. 2002. Cytogeography and reproduction of the *Paspalum simplex* polyploid complex. Plant Syst. Evol. 236:99–105. doi:10.1007/s00606-002-0237-6

Vogel, K.P., and B.L. Burson. 2004. Breeding and genetics. In: L.E. Moser, et al., editors, Warm-season (C_4) grasses. Agron. Monogr. 45. ASA, CSSA, and SSSA, Madison, WI. p. 51–95. doi:10.2134/agronmonogr45.c3

Weiler, R.L., M. Dall'Agnol1, C. Simioni, K.C. Krycki, N. Dahmer, and D. Guerra. 2017. Determination of the mode of reproduction of bahiagrass hybrids using cytoembryological analysis and molecular markers. Rev. Bras. Zootec. 46:185–191. doi:10.1590/s1806-92902017000300002

Weiler, R.L., M. Dall'Agnol1, C. Simioni, K.C. Krycki, E.A. Pereira, J. Medianeira Machado, and E.A. Minski da Motta. 2018. Intraspecific tetraploid hybrids of *Paspalum notatum*: Agronomic evaluation of segregating progeny. Sci. Agric. 75:36–42. doi:10.1590/1678-992x-2016-0354

Worthington, M., C. Heffelfinger, D. Bernal, C. Quintero, Y.P. Zapata, J.G. Perez, et al. 2016. A parthenogenesis gene candidate and evidence for segmental allopolyploidy in apomictic *Brachiaria decumbens*. Genetics 203:1117–1132. doi:10.1534/genetics.116.190314

Zilli, A.L., C.A. Acuña, R.R. Schulz, E.A. Brugnoli, V. Guidalevich, C.L. Quarin, and E.J. Martínez. 2018. Widening the gene pool of sexual tetraploid bahiagrass: Generation and reproductive characterization of a sexual synthetic tetraploid population. Crop Sci. 58:762–772. doi:10.2135/cropsci2017.07.0457

Zilli, A.L., E.A. Brugnoli, F. Marcón, M.B. Billa, E.F. Rios, E.J. Martínez, and C.A. Acuña. 2015. Heterosis and expressivity of apospory in tetraploid Bahiagrass hybrids. Crop Sci. 55:1189–1201. doi:10.2135/cropsci2014.10.0685

Zorzatto, C., L. Chiari, G. De Araújo Bitencourt, C.B. Do Valle, G.O. De Campos Leguizamón, I. Schuster, and M.S. Pagliarini. 2010. Identification of a molecular marker linked to apomixis in *Brachiaria humidicola* (Poaceae). Plant Breed. 129:734–736. doi:10.1111/j.1439-0523.2010.01763.x