Heterosis and Expressivity of Apospory in Tetraploid Bahiagrass Hybrids

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ABSTRACT

Breeding procedures developed for apomictic species are based on the idea of fixing superior hybrids by apomixis. However, scarce information is available about the occurrence of heterosis in apomictic hybrids. The objective was to generate a group of apomixis-segregating tetraploid bahiagrass (*Paspalum notatum* Flüggé) families, evaluate the occurrence of heterosis for a series of agronomic and morphological traits, determine the level of apospory expressivity among hybrids, and estimate the genetic distance among parents and its relationship to heterosis and apospory expressivity. In total, 11 tetraploid families were generated by crossing sexual and apomitic genotypes. The segregation for mode of reproduction was analyzed using a random amplified polymorphic DNA (RAPD) marker linked to apospory in bahiagrass, and the level of apospory expressivity was determined using embryo sac observations. The genetic distances between parents were determined using inter-simple sequence repeat (ISSR) markers. The ratio between sexual and aposporic hybrids varied from 1:1 to 7:1 among families. Discontinuous variation for apospory expressivity was observed in the hybrids, with either low or high levels being exhibited. Mid-parent, high-parent, and standard heterosis was observed for all evaluated characteristics. The level of heterosis was dependent on the combination of parents involved and also on the specific trait. There was a low correlation between genetic distances among parents and initial growth and the level of apospory expressivity. The occurrence of heterosis and the segregation and expressivity for apospory were highly dependent on the combination of sexual and apomictic parents.

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Abbreviations: ISSR, inter-simple sequence repeat; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; SSR, single sequence repeat.

Warm-season forage grasses have become economically very important considering their value for beef cattle production in the tropics and subtropics. This occurrence is especially noticeable in Brazil, which has one of the world's largest beef production and exportation industries, where most of the beef production is centered around cultivated apomictic forages (Jank et al., 2014). Among warm-season apomictic forage grasses, the species with the highest economic values belong to the genera *Brachiaria*, *Panicum*, *Cenchrus*, and *Paspalum* (Moser et al., 2004; Miles, 2007; Blount and Acuña, 2009; Jank et al., 2014).

Genetic improvement of these species has traditionally been accomplished by selecting natural apomictic variants or genotypes, which in all documented cases were polyploid (Vogel and Burson, 2004; Miles, 2007). These genotypes are usually highly stable and their superior characteristics are maintained across cropping cycles. This breeding method is especially appropriate for highly polymorphic species. Recent population research has indicated that *Paspalum* species contained a rich diversity, which is mainly present among populations as the result of site-specific colonization of novel apomictic genotypes (Brugnoli et al., 2014).

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Hybridization in apomictic species becomes possible once rare sexual variants are discovered or when sexual tetraploids are created by doubling the chromosome number of naturally occurring sexual diploid genotypes (Vogel and Burson, 2004). The progenies from crossing sexual and apomictic genotypes are usually highly variable and segregate for apomixis. The primary goal for using hybridization in apomictic species is to fix superior hybrids by apomixis. Extensive research has been conducted to define the inheritance of apomixis in forage grasses and convincing evidence was found for a dominant Mendelian factor controlling apomixis (Miles, 2007). However, scarce information is available about the occurrence of heterosis in these species and the possibility of obtaining superior and highly apomictic hybrids.

Bahiagrass is a dominant species of rangelands in South America and is used as a cultivated pasture around the world (Gates et al., 2004). It is particularly important for the beef-cattle industry in the Coastal Plain region of southeastern United States, where it is one of the primary cultivated forage species (Blount and Acuña, 2009). This species has different ploidy levels and linked reproductive characteristics. Sexual, cross-pollinated diploid ($2n = 2x = 20$) genotypes have been found naturally located in northeastern Argentina (Burton 1955, 1967). The most commonly found bahiagrass cytotype is tetraploid ($2n = 4x = 40$), which is typically distributed across South and Central America and reproduces by aposporous apomixis (Gates et al., 2004).

Bahiagrass has been used as a model for genetic research and breeding apomictic forage grasses because pioneering research described a lack of observed variability in progeny derived from a tetraploid ecotype and the possibility of apomictic reproduction in the species (Burton, 1948; Ortiz et al., 2013). Colchicine-induced sexual tetraploid bahiagrass genotypes were first created by Forbes and Burton (1961), making it possible to hybridize and select at the tetraploid level. However, the genetic control of apomixis had not been well defined until Martínez et al. (2001) performed an in-depth analysis of the inheritance of apospory in bahiagrass, concluding that a single, dominant allele with a pleiotropic lethal effect and incomplete penetrance controls apospory development. More recently, Quesenberry et al. (2010) created a large group of sexual tetraploid genotypes from the diploid cultivar Tifton 9 through tissue culture with the objective of initiating a breeding program for tetraploid bahiagrass in the southeastern United States. These novel induced sexual genotypes were successfully crossed with apomictic genotypes and their progeny showed a wide range of variability for agronomic traits, such as cold tolerance and cool-season growth (Acuña et al. 2009, 2011). However, the occurrence of significant heterosis in the apomictic hybrids was not researched. Furthermore, variability for apospory expressivity was identified among hybrids (Acuña et al., 2011). Variable levels of apospory expressivity could reduce the probability of fixing hybrids exhibiting heterosis for traits of interest by apomixis. Thus, more research is needed to better define the expressivity of apospory in bahiagrass and its genetic control.

Considering the importance of heterosis for breeding apomictic species, new techniques for exploiting nonadditive genetic effects have been proposed (Miles, 2007). In addition, the possibility of predicting heterosis in apomictic species would be desirable because it would result in a more efficient and streamlined process of generating superior hybrids that can be fixed by apomixis. The value of using molecular markers for the identification of heterotic groups has been tested in sexual crops with variable results. Missaoui et al. (2006) identified two heterotic groups named upland and lowland in switchgrass (*Panicum virgatum* L.) using RFLP markers, and the resulting hybrids between these two groups exhibited heterosis for biomass yield (Martinez-Reina and Vogel, 2008). Moreover, Reif et al. (2003) were able to group tropical maize (*Zea mays* L.) populations in different heterotic groups for grain yield using single sequence repeat (SSR) marker analysis. In contrast, alfalfa (*Medicago sativa* L.) hybrids resulting from crossing parents selected based on their genetic distances were not different for forage yield (Kidwell et al., 1999). The objectives of this research were to (i) generate a group of apospory-segregating families by crossing sexual and apomictic bahiagrass genotypes, (ii) evaluate the occurrence of heterosis for a group of agronomic and morphological traits, (iii) determine the level of apospory expressivity among hybrids, (iv) estimate the genetic distance among sexual and apomictic parents using ISSR markers, and (v) evaluate the relationship of genetic distance with heterosis, the proportion of aposporic hybrids, and the level of apospory expressivity.

MATERIALS AND METHODS

Plant Material and Crosses

The origin and mode of reproduction of each parental genotype used in this study can be found in Table 1. Crosses were made between a group of sexual and apomictic bahiagrass genotypes in January 2010. Each accession number was given to individual genotypes in Table 1. A day before anthesis, rooted culms of sexual plants bearing panicles were collected and immediately placed in a 1-L container with water. These culms were then placed in an artificial fog chamber that started misting the next day 2 h before sunrise. Anthesis occurred around sunrise and the high level of air humidity prevented anther dehiscence. Sharp, pointed tweezers were used to remove the anthers. Emasculated inflorescences of the sexual plants were dusted with the desired pollen from the apomictic male parent. Inflorescences were covered with glassine bags after pollination to prevent contamination with pollen from undesired sources. The containers were then placed in a shaded corner of the glasshouse where they remained for 30 d after pollination. Inflorescences from the sexual parents were then manually threshed and seed were separated using a seed blower.

Table 1. Identification, mode of reproduction, and origin of tetraploid genotypes of *Paspalum notatum* used in this experiment.

Accession	Reproduction mode	Origin
SWSB	Sexual	Sexual white stigma bahiagrass, derived from hybrids originally generated by G.W. Burton by crossing an induced tetraploid plant with an apomictic natural tetraploid bahiagrass with white stigmata, WSB (Burton and Forbes, 1961) and then introduced to Argentina in 1979 (Quarin et al., 1984).
Q4205	Sexual	Obtained by self-pollination of SWSB (Quarin et al., 2003).
C4–4x	Sexual	Colchicine-induced tetraploid from a diploid plant collected at Cayasta, Santa Fe, Argentina (Quarin et al., 2001).
C&A1556	Apomictic	Route 35, Km. 364 near Fortin Olavarría, Buenos Aires, Argentina.
Q3838	Apomictic	Riachuelo, Corrientes, Argentina.
V14327	Apomictic	Capivari, RS, Brazil.
Q3775	Apomictic	Tamaulipas, Mexico.
Q4064	Apomictic	Saladas, Corrientes, Argentina.
SV2893	Apomictic	El Huayo, Department of Cajamarca, province of Cajabamba, Peru.
Q3776	Apomictic	Villa Tunari, Chapare region, Bolivia.
Q4294	Apomictic	Los Algarrobos, Santa Rosa de Calamuchita, Córdoba, Argentina.
B229	Apomictic	Itaqui, RS, Brazil.
Argentine	Apomictic	USDA PI 148996. Imported from the United States in 2010 (granted by Dr. Ann Blount, University of Florida).

Classification for Mode of Reproduction

The hybrid seed were scarified using 98% sulfuric acid for 10 min, washed, and germinated in sterile germination media. Seedlings with three leaves were transplanted to 100-mL cell seedling flats. After 30 d the plants were transplanted into the field on 1-m centers in October 2011. The progeny from each cross was referred to as a family, and a capital letter was given to identify each family (Table 2). Each genotype resulting from a cross was named hybrid (Table 2). The field was located within the Campus of Universidad Nacional del Nordeste, Corrientes, Argentina.

Genomic DNA from a sample of the tiller's apical meristem of each hybrid was extracted using the methodology described by Brugnoli et al. (2014). Genomic DNA integrity and concentration was estimated using known patrons in 1% w/v agarose gel in 1× TAE (40 mM Tris-HCl, 5 mM sodium acetate, 0.77 mM EDTA, pH 8.0) at 40 V for 1 h. DNA samples were visualized under ultraviolet light and photographed with GelDoc-It Imaging System (UVP, LLC) after staining with ethidium bromide (1 $\mu g\ mL^{-1}$) for 30 min. DNA samples were adjusted to 10 ng μL^{-1} for their use in polymerase chain reaction (PCR) amplifications.

Hybrids were initially classified as aposporic or sexual using a RAPD primer (UBC243: GGGTGAACCG), which generated a specific marker of 377 bp completely linked to the apospory trait in bahiagrass (Martínez et al., 2003). The PCR protocol was the same as used by Martínez et al. (2003). The PCR products were separated by electrophoresis in 2% w/v agarose gel in 1× TAE (40 mM Tris-HCl, 5 mM sodium acetate, 0.77 mM EDTA, pH 8.0) at 70 V for 3 h. The PCR amplifications were visualized and photographed as described above. The plants showing the specific marker were classified as aposporic, and the rest as sexual (Fig. 1). Previous studies have shown that all the sexual female parents did not have this molecular marker, while all the apomictic parents did. The DNA samples from all parents were used as positive (apomictic parents) or negative (sexual parents) controls.

Table 2. List of crosses made between sexual and apomictic bahiagrass genotypes, total number of hybrids, number of sexual and aposporic hybrids, and the ratio between sexual and aposporic hybrids.

Cross combination		Hybrids				
Sexual female parent	Apomictic male parent	ID	Total	Sexual	Aposporic	Ratio sexual: aposporic
SWSB	B229	A	42	37	5	7.4:1
SWSB	Q4064	B	46	32	14	2.3:1
SWSB	SV2893	C	48	38	10	3.8:1
SWSB	Q4294	D	49	40	9	4.4:1
SWSB	V14327	E	50	25	25	1:1
SWSB	Q3775	F	50	36	14	2.6:1
Q4205	Q3776	G	50	42	8	5.3:1
Q4205	CyA1556	H	50	43	7	6.1:1
Q4205	Q4064	I	50	35	15	2.3:1
C4-4x	Q3838	J	39	29	10	2.9:1
C4-4x	Q4294	K	50	41	9	4.6:1
Total			524	398	126	3.2:1

Agronomic and Morphological Characterization of Aposporic Hybrids

The aposporic hybrids (individual genotypes) and their parents were vegetatively propagated in a greenhouse, generating four clonal replicates for each genotype. Hybrids, their parents, and the cultivar Argentine were planted into the field on 1-m centers in a randomized complete block design with four replications. A border row of bahiagrass plants was planted around the plot. The experiment was planted near the city of Corrientes, Argentina (27°38′ S, 58°44′ W) on 22 November 2012. The soil type was classified as Argiudoll. The initial growth was estimated on 9 April 2013 using a 1-to-5 scale, where 1 represented the plants exhibiting the lowest amount of aboveground growth, and 5 represented the plants with the highest amount of growth. Plants were then cut to approximately 10-cm stubble height on 10 April 2013. Fall regrowth was estimated on 15 May using the scale described above. Frost resistance was visually estimated on 30 August 2013 after one frost event occurred on 27 August, with temperature reaching −0.2°C, using a 1-to-5 scale, where 1 = the least frost resistant, and 5 = the most resistant plant. Spring regrowth was estimated on 22 October 2013 and summer regrowth on 28 January 2014 using the same scale described for initial growth.

Growth habit was estimated by measuring plant diameter and height. Plant diameter (cm) was determined using the average between the longest and shortest diameter of a given plant while plant height (cm) was measured from the base of the plant to the top of the canopy. These two variables were measured in November 2013. Biomass yield was determined by cutting individual plants at 8-cm stubble height on 5 December 2013. The fresh

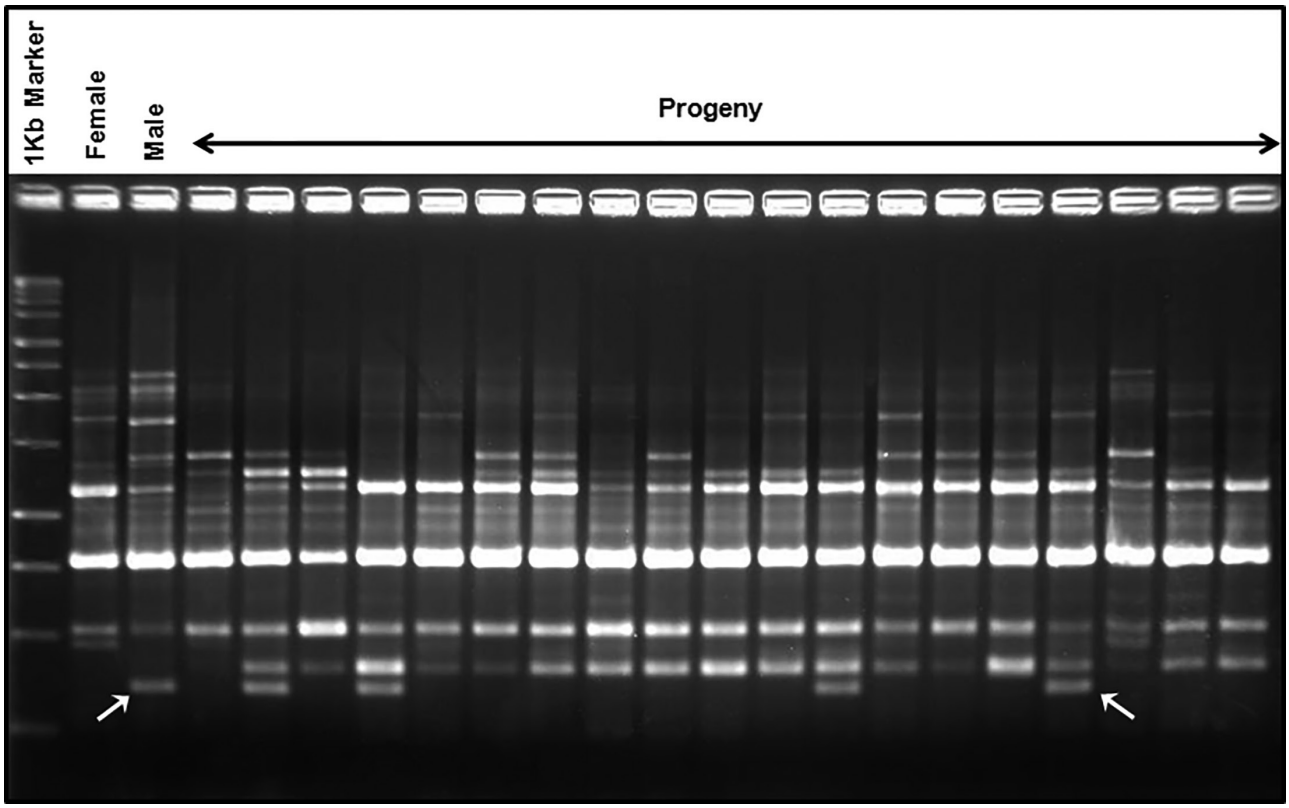

Figure 1. Electrophoretic pattern generated using the UBC243 random amplified polymorphic DNA primer on progeny of an apospory-segregating tetraploid bahiagrass population. Arrows indicate the presence of the apospory-linked molecular marker (377 bp).

weight of the harvested material was recorded and a subsample was collected and dried at 60°C for 48 h. The dry subsample was weighed (g) and the amount of harvested biomass was calculated.

Leaf width (mm) was measured at the widest point of the leaf blade on the first fully expanded leaf. Leaf-blade length (cm) was also measured on the first fully expanded leaf. Both leaf traits were measured from three different tillers in each plant during the first week of February 2014. The flowering stem height (cm) was assessed from the soil level to the axis of the basal raceme. The length of the basal raceme (cm) was also measured. Both traits were measured using three inflorescences from each plant during the first week of February 2014.

Apospory Expressivity

Inflorescences of each aposporic hybrid were fixed in FAA (70% ethanol, 37% formaldehyde, and glacial acetic acid in the ratio 18:1:1) at anthesis. Pistils were dissected out and clarified using the method described by Young et al. (1979) with the following modifications: after about 24 h in FAA, pistils were dissected out and transferred to ethanol 70% for 24 h. Pistils were passed through a series of dehydration and clearing solutions (250 µL of each solution in 1.5-mL tubes) as described below: 3% hydrogen peroxide (H_2O_2) (2 h); 50% ethanol (30 min); 70% ethanol (30 min); 95% ethanol (30 min); 100% ethanol (30 min) twice; methyl salicylate/Ethanol 50:50 v/v (30 min); methyl salicylate/ethanol 75:25 v/v (30 min); methyl salicylate/ethanol 85:15 v/v (30 min); 100% methyl salicylate (about 12 h). Ovules were observed using a differential interference contrast microscopy, photographed with a Leica EC3 camera.

A minimum of twenty-five pistils from at least two inflorescences of each plant were observed. Ovules bearing a single embryo sac containing the egg apparatus, the binucleated central cell, and a mass of antipodals at the chalazal end were classified as meiotic (Fig. 2a). In contrast, ovules bearing multiple or single embryo sacs with the egg apparatus, the central cell, no antipodals, and variable size and position, were classified as aposporous (Fig. 2b). Ovules were classified as mixed aposporous-meiotic when both reduced and unreduced embryo sacs were present in the same ovule. The level of apospory expressivity (LAE) was calculated as follows:

$$LAE = [(NAO + NMO)/TNO] \times 100$$

where NAO was the number of ovules with aposporous embryo sacs, NMO was the number of ovules with mixed aposporous-meiotic embryo sacs, and TNO was the total number of analyzed ovules.

Genetic Distance Between Parents

The determination of the genetic distance between parents was performed using ISSR markers. DNA extraction from each parent was performed as described above. Ten ISSR primers were used for PCR amplification following the protocol described by Cidade et al. (2008) with annealing temperature modified according to the primer. The PCR products were separated by electrophoresis in 2% w/v agarose gel in 1× TAE (40 mM Tris-HCl, 5 mM sodium acetate, 0.77 mM EDTA, pH 8.0) at 70 V for 3 h. Gels were stained with ethidium bromide

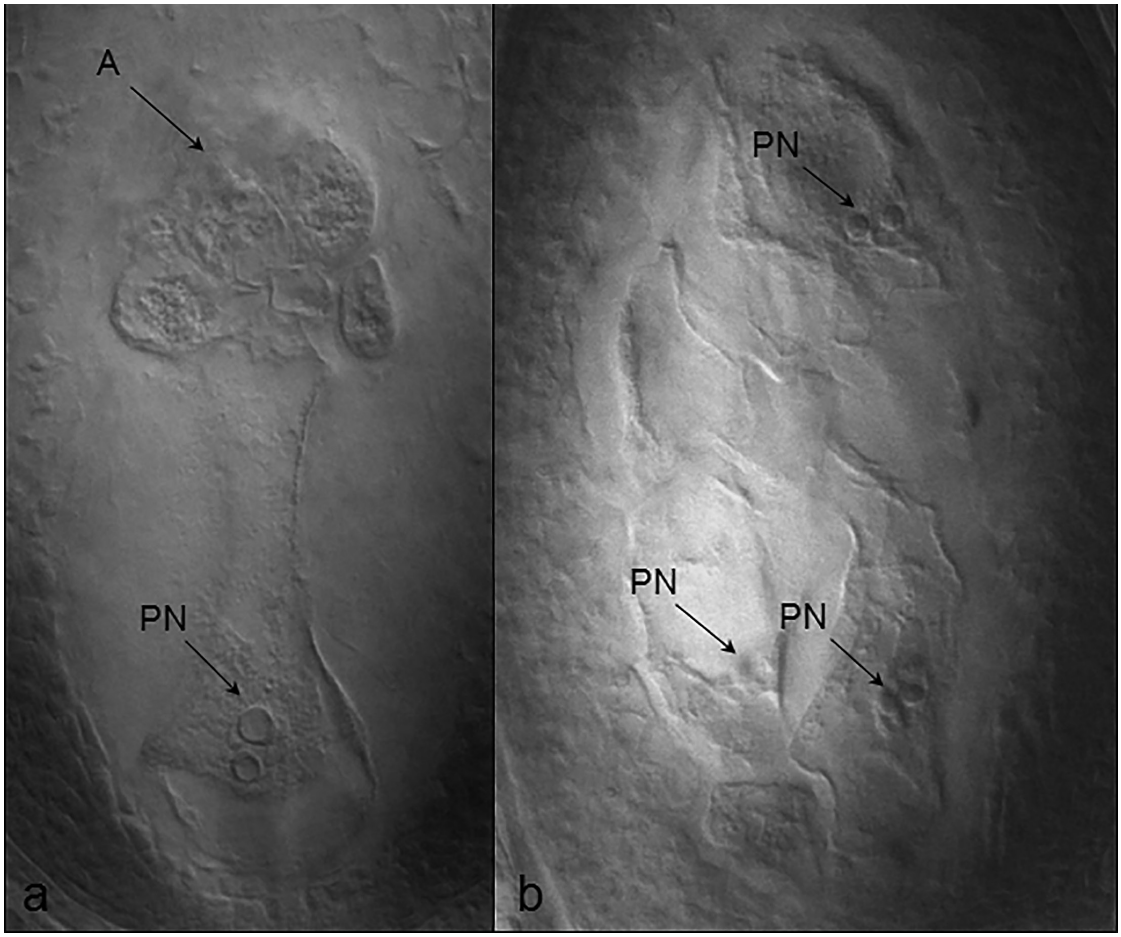

Figure 2. Embryo sacs of tetraploid bahiagrass: (a) A meiotically derived embryo sac containing the antipodals (A) and the polar nuclei (PN); (b) A group of three aposporous embryo sacs within the same ovule.

(1 μg mL^{-1}) and fragments were visualized under ultraviolet light, photographed, and stored for further analysis with Gel-Doc-It Imaging System. The relationship between the genetic distance between parents and the occurrence of heterosis, the proportion of aposporic hybrids and apospory expressivity among hybrids was evaluated.

Efficiency of the Crossing Procedure

The efficiency of the crossing technique considering the possibility of self-pollination in tetraploid female parents was evaluated using ISSR markers with a sample representing 21% (110 plants) of the generated progeny. This sample contained 10 plants from each family. DNA extraction and PCR protocol were as described above. An initial screening for ISSR markers only present in the apomictic male parent was performed using the sexual and apomictic parents involved in each cross. The progeny was evaluated for the presence of those markers only present in the apomictic parent. A minimum of two ISSR markers per plant were considered to classify the progeny as a true hybrid.

Statistical Analyses

Agronomic and morphological characteristics data were analyzed using Info-Gen software (Balzarini and Di Rienzo, 2004) as a randomized complete block design. Means, coefficients of variation, standard deviation, analysis of variance (ANOVA), and mean separations by the LSD test were calculated.

The proportion of hybrids exhibiting positive or negative midparent, high-parent, and standard heterosis were calculated for all traits. Heterosis values were calculated as mean differences, which permits the use of mean comparison statistics such as the LSD for comparing mean differences between hybrids, parents, and a control cultivar or standard.

Midparent heterosis (MPH) was calculated using the following formula:

$$MPH = (F_1 - MP)$$

where F_1 was the performance of a hybrid, and MP was the average performance of parents (parent 1 + parent 2)/2.

High-parent heterosis (HPH) was calculated using the following formula:

$$HPH = (F_1 - HP)$$

where F_1 was the performance of a hybrid, and HP was the performance of the high parent.

Standard heterosis (StH) was calculated using the following formula:

$$StH = (F_1 - St)$$

where F_1 was the performance of a hybrid, and St was the performance of the cultivar Argentine used as standard.

DNA amplification profiles obtained with ISSR molecular marker were introduced in a binary matrix, scoring the presence of the marker as 1, and the absence as 0. This matrix was analyzed using Info-Gen software. Genetic distance among parents was estimated using the Jaccard's dissimilarity coefficient (Kosman and Leonard, 2005).

The relationship between genetic distance among parents, and the occurrence of heterosis, apospory expressivity, and the segregation for apospory among hybrids was estimated with the Person's correlation coefficient.

RESULTS

A group of 524 tetraploid hybrids, corresponding to 11 families, were generated by crossing artificially created sexual and naturally occurring apomictic genotypes of bahiagrass. Each family consisted of between 39 and 50 hybrids (Table 2).

The efficiency of the crossing technique was evaluated using 19 ISSR primers in a sample of 110 plants (10 plants per family). Only two plants (1.8%) did not have markers that were only present in the male parent and were considered products of self-pollination. Thus, the efficiency of the crossing method was 98.2%.

The RAPD marker completely linked to apospory was amplified in 126 of the 524 hybrids, leading to their initial classification as aposporic (Table 2). An overall ratio of 3.2 sexual to 1 aposporic hybrid resulted from these crosses, however, the ratio varied between 7.4:1 and 1:1 among families (Table 2).

Heterosis in Aposporic Hybrids

Agronomic and morphological traits were measured on 112 out of the 126 aposporic hybrids together with their parents and Argentine bahiagrass. Significant differences among families, their parents, and Argentine were observed for all evaluated traits (Table 3). Based on the coefficients of variation, the most variable traits were biomass yield, and summer and fall regrowth (Table 3).

The number of families having hybrids showing midparent heterosis varied from two for flowering stem height and leaf blade length to 11 (all families) for plant height (Fig. 3). The proportion of hybrids exhibiting midparent heterosis was also variable among families for all traits, with the maximum proportion ranging from 14% for leaf blade length to 62.5% for spring regrowth and biomass yield (Fig. 3).

The number of families containing hybrids that exhibited high-parent heterosis varied from zero for leaf-blade length to five for initial growth. The maximum proportion of statistically superior hybrids per family was 62.5% for biomass yield (Fig. 4). A percentage of hybrids were significantly inferior to the inferior parent in each family depending on the analyzed characteristic with the maximum proportion ranging from 10% for biomass yield to 29% for raceme length (Fig. 4).

When comparisons were made between hybrids and Argentine bahiagrass, the number of families containing superior hybrids varied from zero for summer regrowth, plant diameter, leaf-blade length, flowering stem height, and raceme length to six for leaf-blade width and frost resistance (Fig. 5). The maximum proportion of superior hybrids was 50% for frost resistance (Fig. 5). The highest proportion of hybrids significantly inferior to Argentine bahiagrass varied from 17% for frost resistance to 100% for biomass yield, summer and fall regrowth, plant diameter, and leaf-blade length (Fig. 5).

Apospory Expressivity

Variable expressivity for apospory was observed among 96 hybrids out of the 126 initially classified as aposporic using RAPD marker completely linked to apospory. The maximum range of variation was found with hybrids characterized by the presence of aposporous embryo sacs in 100% of their ovules to hybrids bearing only meiotically derived embryo sacs in all their ovules. Dividing the percentage of apospory expressivity among the hybrids in 11 fractions (Fig. 6) revealed that 28% of the hybrids were in the lowest fraction, which included those hybrids that did not have ovules bearing aposporous embryo sacs. Low levels of expressivity (between 1 and 20%) were observed for 12% of hybrids, only 1% of the hybrids fell between 21 and 50% expressivity, 27% of the hybrids exhibited between 51 and 80% expressivity, and 32% were in the upper fractions (between 81 and 100% expressivity).

Genetic Distances among Parents and its Relation with Hybrid Behavior

A total of 112 loci were amplified, and 89 (79%) were identified as polymorphic (Table 4). An example of the obtained electrophoretic pattern can be observed in Fig. 7. Genetic distances between sexual and apomictic parents varied from 0.36 to 0.58 (Jaccard dissimilarity coefficient). A positive correlation was found between genetic distances among parents and midparent heterosis for initial growth ($r = 0.3$; $p = 0.003$). No significant correlations were observed between genetic distances and heterosis for the remainder of the morphological and agronomic traits.

The proportion of aposporic hybrids in each family (Table 2) was not significantly correlated with the genetic distance between parents. However, the level of apospory expressivity in aposporic hybrids was found to be correlated with the genetic distances between parents ($r = 0.23$; $p = 0.02$).

Table 3. Mean and coefficient of variation for a series of agronomical and morphological traits measured on 112 aposporic bahiagrass hybrids from 11 different sexual apomictic bahiagrass populations (A–K, parents identified in Table 2).

	Initial growth		Fall regrowth		Frost resistance		Spring regrowth		Summer regrowth		Biomass		Height		Diameter		Leaf blade length		Raceme length		Flowering stem height		Leaf blade width	
	Mean†	CV	Mean†	CV	Mean†	CV	Mean†	CV	Mean†	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
											g		cm		cm		cm		cm		cm		mm	
Standard	2.3	39	3.5	15	2.7	17	3.5	15	4.3	18	334.4	25	37.1	11	83.6	16	36.8	11	11.8	10	62	12	65	13
SP‡-ABCDEF	2.5	23	4	20	3.8	33	4	20	3.5	17	365.9	52	39	39	63.1	22	40.5	23	10.7	11	59.5	6	60	20
AP§-A	2.3	43	2.5	23	2.5	23	3	0	3.3	29	226.5	62	26.5	24	96	4	31.8	12	11.3	9	60.6	1	81	20
A	2.5	47	3.4	37	3.6	20	3.3	27	3	29	240	39	38.9	19	63.2	22	35.9	16	10.5	15	56.8	20	62	13
AP-BI	2.5	23	3	27	1.8	29	3	0	4	29	357	61	29.3	9	98.8	12	34.3	9	12.2	7	61.8	19	81	9
B	3.1	30	3.4	28	2.8		3.3	30	3.3	28	351	46	35.5	26	77.2	26	31.3	17	10.7	16	55.2	20	70	13
AP-C	3	0	3.3	17	3	0	3.3	17	3.7	31	321.1	127	27.3	52	97.8	14	30.1	17	11.9	3	60.6	20	76	13
C	2.7	40	3.2	39	3	29	3.1	42	3.7	35	325	52	34.5	32	62.2	39	35.8	20	10.4	24	59.6	25	71	15
AP-DK	2.3	22	2.7	23	2.7	18	2.3	18	3.3	15	149	31	23.8	22	75.6	20	35.5	5	11.9	5	62.5	8	72	14
D	2.8	35	3.1	33	2.8	26	3.2	33	3.4	27	320	29	36.3	31	62.3	35	38.4	12	11.2	11	61.6	13	64	14
AP-E	1.8	29	1.5	38	2.3	22	1.3	40	1	0	36.8	–	10	28	42.5	22	16.4	–	6.3	–	38.7	–	66	–
E	3.2	33	2.6	42	2.6	46	2.8	39	2.3	40	214.6	56	26.1	30	48.8	23	26.6	24	9.8	14	55.9	19	65	18
AP-F	2.5	23	3	0	2.3	22	3	0	3.5	17	276.7	8	23.8	21	96.4	10	25.9	8	13.8	39	60.3	8	77	4
F	3.1	31	3.3	27	2.9	22	3.4	29	3.4	27	318.1	51	33.1	28	75.4	27	32.7	22	9.8	14	55.9	18	67	14
SP-GHI	4	54	4.3	13	4.7	12	3.7	16	3.3	17	222.8	36	47	6	48	19	41.1	10	8.9	15	50.4	7	64	11
AP-G	2.8	46	3.3	29	2	0	2.3	18	3.5	17	187	53	21.5	31	83.8	21	28.6	19	11.5	14	60	13	73	15
G	3.2	37	3.4	27	2.9	17	3.9	24	3.6	28	356.2	43	39.9	23	72.4	20	36.9	13	10.7	11	59	10	70	14
AP-H	1.5	38	1.7	35	2	50	2.3	25	1.7	35	223.7	104	26.3	18	60.7	17	24.3	43	9.5	2	49.8	8	49	9
H	3.2	35	3.5	31	3.3	20	3.5	26	3.2	24	304.6	41	41.6	23	61.2	25	38.3	15	10	20	55	18	65	13
I	3.2	28	3.5	27	3.4	24	3.6	33	3.6	31	372.2	45	45.3	28	61.6	29	37.6	13	11.2	13	60.6	15	66	15
SP-JK	3.3	17	2	0	2.7	22	1.7	35	1	0	233.2	127	19.3	13	52.2	15	–	–	–	–	–	–	–	–
AP-J	1.8	29	1.5	38	2.5	40	2.5	23	1.5	38	84.5	40	12.3	29	86.4	13	20.5	19	3	95	11	98	47	8
J	2.5	40	1.7	38	3.2	20	2.2	36	1.8	44	100.5	57	19.7	40	59.8	34	21.2	28	10	20	55	18	45	26
K	2.5	33	2.3	37	3.2	13	2.7	38	2.4	43	186.1	57	28.3	31	71	28	27.9	17	9.7	21	45.5	32	60	13
LSD 0.05	1.3		1		0.8		1		1.2		166.2		9.5		17.9		7.4		2.2		12.4		12	
CV	36		37		30		35		38		55		36		32		24		21		25		17	

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

‡ SP, sexual parent for the indicated families.

§ AP, apomictic parent for the indicated families.

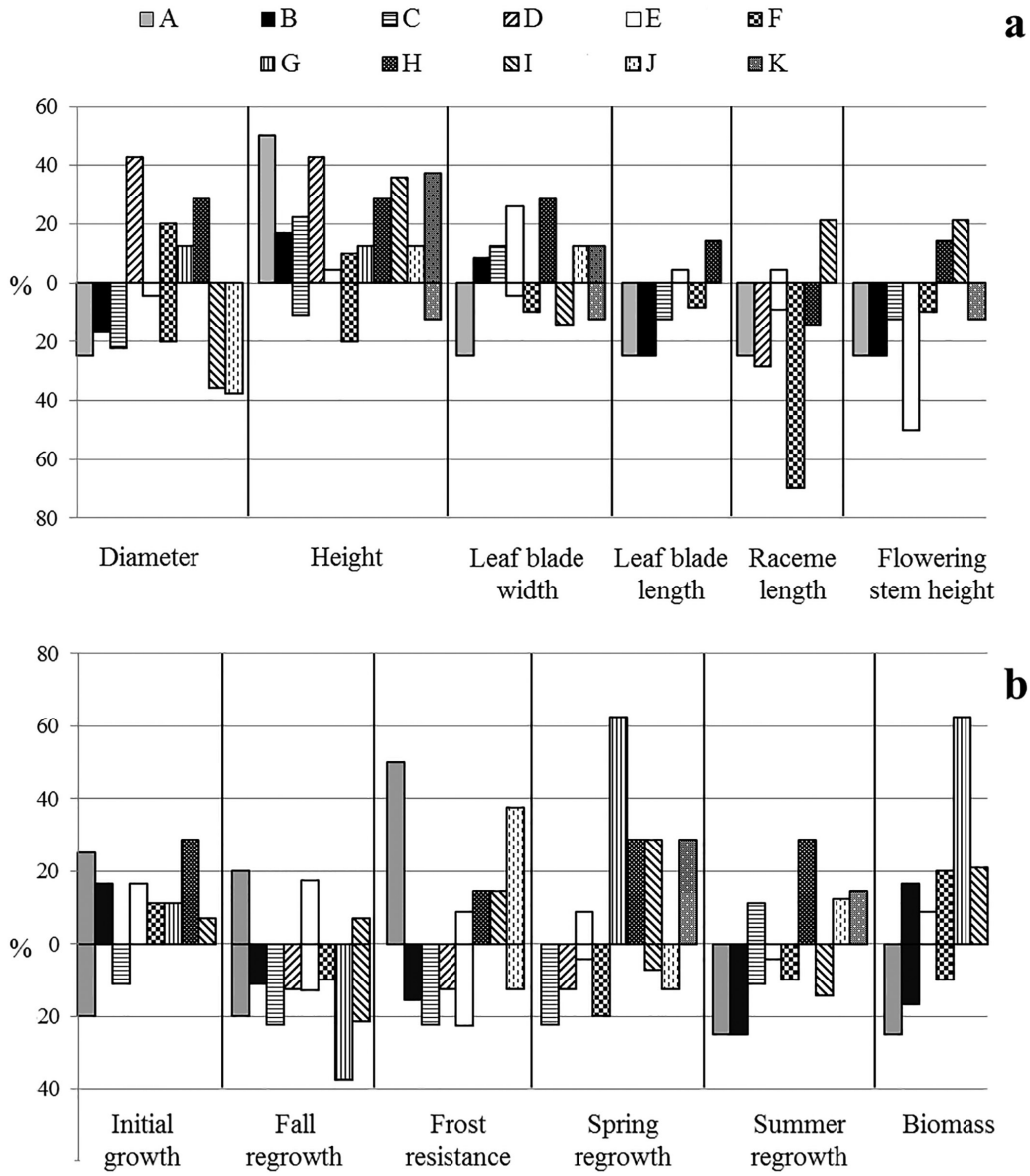

Figure 3. Proportion of aposporic bahiagrass hybrids from 11 different sexual × apomictic bahiagrass populations (A–K) exhibiting mid-parent heterosis for a series of 12 (a) morphological and (b) agronomic characteristics. Hybrids that were not statistically different from the respective comparison are not represented in the bar graph.

DISCUSSION

The occurrence of heterosis defines the usefulness of hybridization for cultivar development in apomictic species. In this research, we evaluated the possibility of generating superior and highly aposporic bahiagrass hybrids in comparison to their parents and a commercial cultivar. The expressivity of apospory was also evaluated considering its importance for generating stable apomictic cultivars. The significance of the genetic distance between parents was also evaluated considering the possibility of predicting hybrid vigor, the proportion of aposporic hybrids, and the level of apospory expressivity in the resulting progeny.

Segregation and Variable Expressivity of Apospory

Assuming that a dominant Mendelian factor is controlling apomixis in bahiagrass, a 1:1 segregation ratio between sexual and apomictic hybrids is expected. However, a higher-than-expected number of sexual hybrids was observed in most of the analyzed families. This phenomenon was repeatedly observed in hybrids from crosses between sexual and apomictic tetraploid genotypes of bahiagrass that were segregating for reproduction mode and was attributed to the presence of a lethal genetic factor with incomplete penetrance associated with the apospory-controlling locus (Martínez et al., 2001; Stein et al., 2004; Acuña et al., 2011). Furthermore, there is evidence indicating that the low transmission of apospory in bahiagrass is related to the

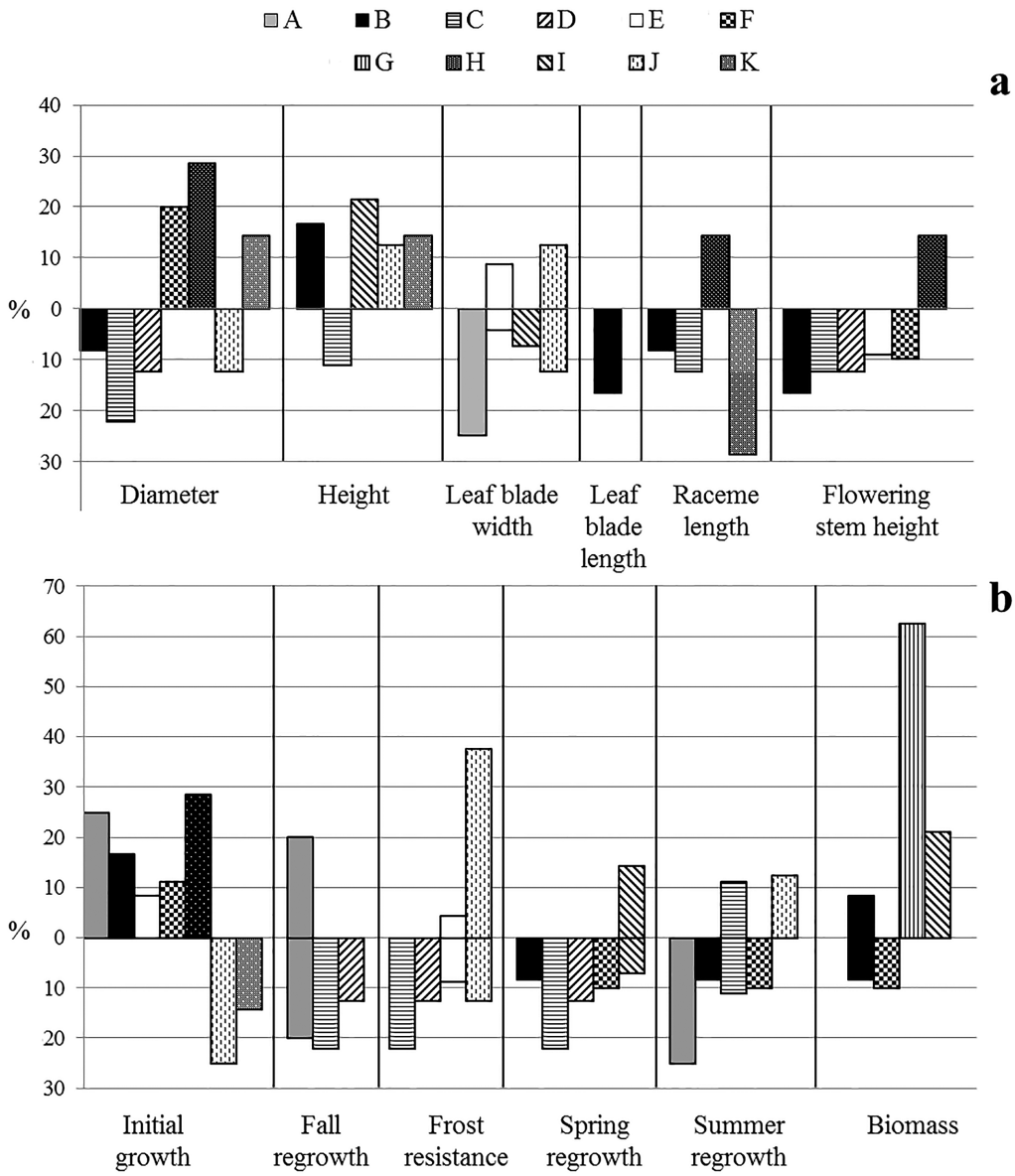

Figure 4. Proportion of aposporic bahiagrass hybrids from 11 different sexual × apomictic bahiagrass populations (A–K) exhibiting high parent heterosis for a series of 12 (a) morphological and (b) agronomic characteristics. Bars below the abscissa indicate the proportion of hybrids that were significantly inferior to the inferior parent.

presence of an inversion in the chromosomal region where the apospory-controlling locus is located (Stein et al., 2004; Podio et al., 2012). Equal proportions of sexual and aposporic hybrids were observed in Family E (Table 2). This finding indicates that the putative lethal factor may not be present in the male parent used to create Family E.

Segregation ratios for sexual and aposporic hybrids resulting from single female parents were highly variable (Table 2). For example, the segregation ratios varied from 7:1 to 1:1 (sexual/aposporic) when the female parent was SWSB. However, similar ratios were observed when the apomictic male parent was used in crosses with more than one sexual parent (Table 2). This was the case for the apomictic parents Q4064 and Q4294. This is an indication that the segregation for apospory is mainly linked to the apomictic male parent.

It has been proposed that the rate of transmission of apomixis in bahiagrass would decrease as the genetic distances between parents increases due to lack of specific gene interactions between the apospory-controlling genes and the rest of genetic factors involved in the expression of apomixis (Ortiz et al., 2013). However, the absence of a correlation between genetic distances among parents and the proportion of aposporic hybrids in the progeny observed in our research does not support this hypothesis.

Structural differences between aposporous and meiotically derived embryo sacs of mature ovules were analyzed in this study to determine the level of apospory expressivity in the hybrids. Although other techniques are available to evaluate the expressivity of the trait, such as seed analysis by flow cytometry, field progeny test, or progeny

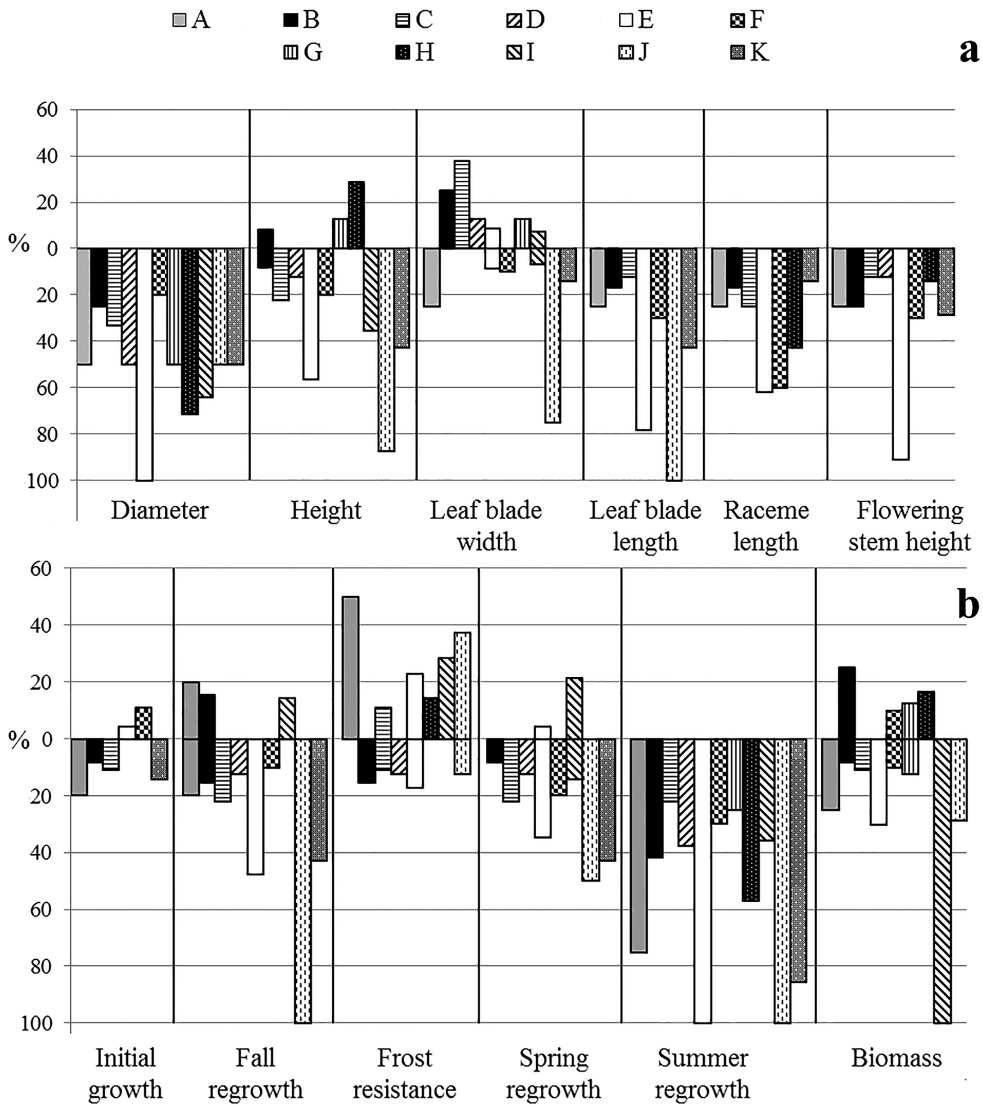

Figure 5. Proportion of aposporic bahiagrass hybrids from 11 different sexual × apomictic bahiagrass populations (A–K) exhibiting standard heterosis for a series of 12 (a) morphological and (b) agronomic characteristics. Hybrids that were not statistically different from the respective comparison are not represented in the bar graph.

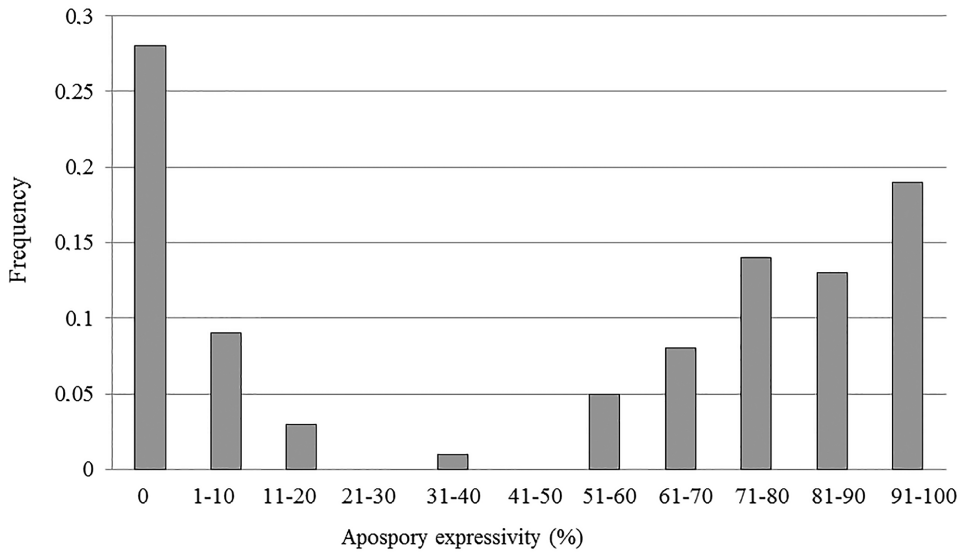

Figure 6. Level of apospory expressivity for a group of 96 tetraploid bahiagrass hybrids from 11 different sexual × apomictic families.

Table 4. Inter-simple sequence repeat molecular characterization of the nine male parents and three female parents: analyzed primers, number of polymorphic loci (PL), total number of loci (TL), proportion of polymorphic loci (PPL), and polymorphism information content (PIC) obtained for each primer.

Primer	PL	TL	PPL	PIC
(AG)8-GC	11	14	79	0.24
(AG)8-T	2	6	33	0.30
(GA)8-TC	9	11	82	0.26
(GA)8-T	7	9	78	0.28
(AC)8-G	7	8	88	0.31
(GA)8-C	12	15	80	0.29
(AG)8-C	9	12	75	0.32
GAG-(AC)7	12	14	86	0.26
CAG-(CA)7	13	15	87	0.26
(AC)8-T	7	8	88	0.24
Total	89	112	79	

test based on molecular markers, embryo sac observations were used because they have been shown to accurately measure the expression of apomixis (Ortiz et al., 1997). Discontinuous variation for apospory expressivity was observed in the hybrid progeny of the 11 families (Fig. 6). The absence of aposporous embryo sacs in all of the analyzed ovules for 28% of the hybrids that possessed the RAPD marker linked to apospory could be the result of a very low expressivity of the apospory locus or because the marker is segregating within the progeny; however, the latter hypothesis is unlikely (Martínez et al., 2003). Hybrids having the apospory RAPD marker could be divided in two groups with high and low expressivity of apospory, respectively. These results were also previously observed by Acuña et al. (2011) in two bahiagrass hybrid populations derived from two cycles of selection for agronomic traits. Similar findings were reported by Aliyu et al. (2010) in diploid populations of genus *Boechera*, which also were characterized by accessions with low or high expressivity of apomixis. These observations could be the result of the presence or absence of a genetic factors controlling expressivity, or they could be related to a dosage effect of an apospory controlling factors. The development of molecular markers linked to this putative additional genetic factor would be very useful for assessing the genetic improvement of bahiagrass at the tetraploid level.

Our results indicate that as the genetic distances between sexual and apomictic parents increases, the level of apospory expressivity in the resulting aposporic hybrids also increases. Although the level of correlation is low, it may be beneficial to select genetically distant parents when making crosses in a breeding program. Considering the importance of this potential relationship, it would be diligent to evaluate its occurrence in other genotypes of the same and other species.

Occurrence of Heterosis in Tetraploid Bahiagrass

The diversity for agronomic and morphological traits contained in the tetraploid germplasm of bahiagrass described in this work can be considered high, which is characteristic of an undomesticated forage species. This high level of diversity indicates the feasibility of genetically improving tetraploid bahiagrass for traits of agronomic importance.

Considering that the objective of manipulating apomixis for cultivar development is to create superior hybrids, which are fixed by apomixis, the occurrence of heterosis at the individual hybrid level should be investigated. Heterosis was observed for all traits, and its occurrence was highly variable among families and traits. Comparisons with Argentine bahiagrass showed that it is possible to generate hybrids that perform significantly better than the most popular tetraploid bahiagrass cultivar in the southeastern United States. Further studies should consider the evaluation of these hybrids growing in swards for comparison to their parents and other commercial cultivars.

With the exception of initial growth, no correlation was observed between the genetic distance between parents and the occurrence of heterosis. The range of genetic distances observed between sexual and apomictic parents was low in this study and is a limitation for the analysis. Considering the importance of predicting the occurrence of heterosis in apomictic species and the ability of other researchers to identify heterotic groups in varying species using molecular markers (Reif et al., 2003; Missaoui et al., 2006), it could be beneficial to continue exploring this relationship in tetraploid bahiagrass by first categorizing parents into different heterotic groups for further study through crossing.

Hybrids F24 and B8 appeared as the most promising genotypes when it was considered for the level of apospory expressivity, ability to grow, frost resistance, and ground cover. There remains a need for further evaluations on seed yield, tolerance to the stress caused by grazing, and the maintenance of a high level of apomixis through several crop cycles.

In conclusion, segregation for apospory in bahiagrass hybrids generated by crossing sexual and apomictic genotypes was highly variable and depended on the combination of parents used. The expressivity of apospory was also variable, both high and low levels were observed, and exhibited a discontinuous pattern when all hybrids were analyzed. This research supports the occurrence of heterosis to be a common phenomenon among aposporic bahiagrass hybrids, and was highly dependent on the parents used and the trait of interest. The high level of diversity, the occurrence of heterosis for most analyzed traits, and the possibility of generating highly aposporic hybrids indicates that it is possible to generate and fix agronomically superior bahiagrass hybrids by apomixis.

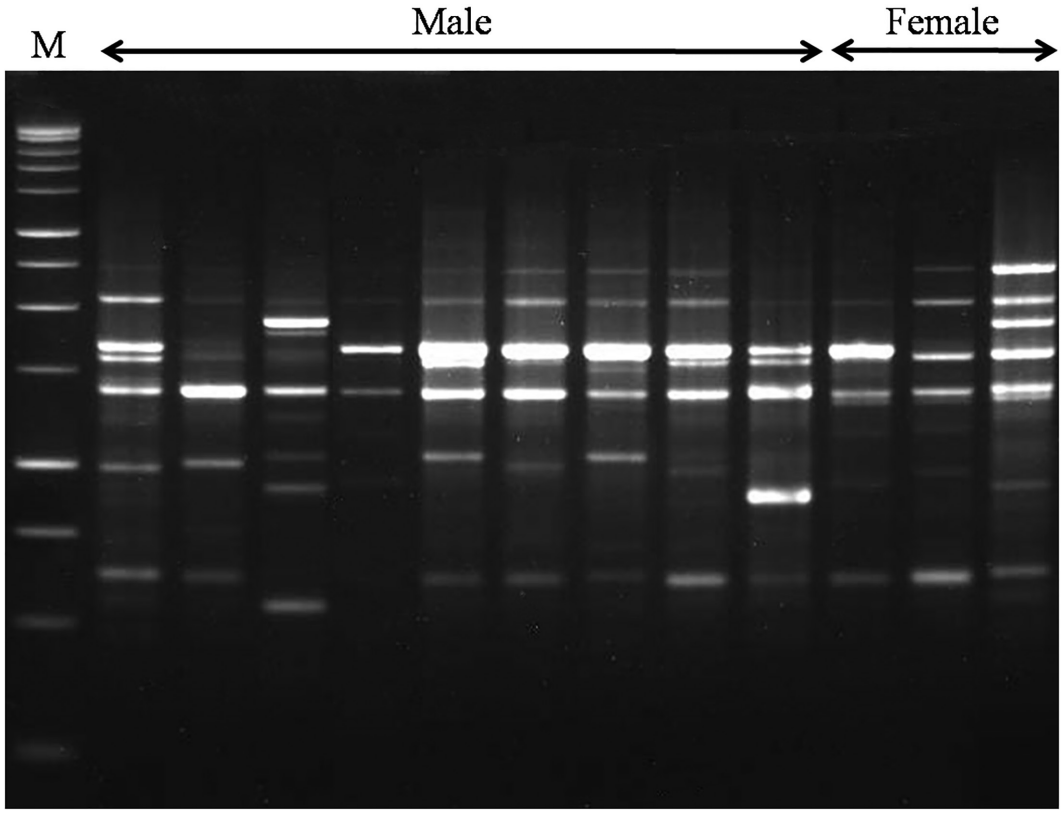

Figure 7. Electrophoretic pattern obtained with the (GA)8-TC inter-simple sequence repeat primer from the analysis of 12 tetraploid genotypes of *Paspalum notatum* used as parents.

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