

Hybridization and heterosis in the *Plicatula* group of *Paspalum*

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Abstract Most forage cultivars released for the genus *Paspalum* belong to a section named *Plicatula*. The species of *Plicatula* are mostly apomictic and consequently the genetic diversity is locked for their genetic improvement. The objectives were to evaluate the crossability, hybrid fertility, heterosis, and genetic distances between apomictic accessions and a sexual genotype of species of *Plicatula* group of *Paspalum*. Crosses were made using 22 apomictic tetraploid accessions belonging to 12 different species as pollen donors, and a sexual tetraploid genotype induced by colchicine from a sexual diploid accession of *P. plicatulum*. Crossability varied between 0 and 16% among crosses. Viable hybrid offspring were recovered from 15 out of 22 crosses. The most successful crosses involved *P. guenoarum*, *P. plicatulum*, *P. chaseanum*, and *P. oteroi*. Fertility of the sampled hybrids varied between 1.6% for the cross involving *P. lenticulare*, and 40.1% for an intraspecific cross (*P. plicatulum*, accession Hojs388). The genetic distance between parents was estimated using amplified fragment-length polymorphism, and it varied between 0.34 and 0.53. There was no correlation between genetic distances and crossability

or fertility of the hybrids. Hybrids from the most numerous families were classified for mode of reproduction using flow cytometric seed analysis. The ratio between sexual and apomictic hybrids varied between 0.6:1 and 1.6:1. A selected group of apomictic hybrids were evaluated for several agronomic traits in the field. Heterosis was observed for frost tolerance and cattle preference. The results indicated that gene transfer via hybridization is possible among several species of *Plicatula*. Superior hybrids for specific traits can be generated and fixed by apomixis.

Keywords Polyploidy · Crossability · Genetic distance · Hybrid vigor · Seed fertility

Introduction

Many cultivated warm-season grasses reproduce asexually by apomixis (Vogel and Burson 2004). This reproductive characteristic offers uniformity and genetic stability across cropping cycles. The most important forage species used for beef-cattle production systems in the tropics and subtropics are apomictic (Jank et al. 2014). The most popular cultivars of apomictic grasses have been developed by direct selection from wild accessions. However, the release of the natural variation locked in the available germplasm of apomictic species becomes possible once sexual plants are found in nature.

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The sexual germplasm is diploid in most species, and chromosome duplication is needed to create a ploidy-compatible sexual plant that can be hybridized with the frequently-tetraploid apomicts (Vogel and Burson 2004). A proportion of the generated progeny inherits apomixis and is usually incorporated in the initial phase of forage evaluation programs. Superior apomictic hybrids are finally released as cultivars. This technique has been used for the genetic improvement of *Cenchrus ciliaris* (Bashaw 1968), *Brachiaria* spp. (Miles et al. 2004), *Panicum maximum* (Muir and Jank 2004), and *Paspalum notatum* (Urbani et al. 2017).

Paspalum is one of the dominant genera in tropical and subtropical grasslands in South America (Zuloaga and Morrone 2005). Its more than 300 species are widespread across the different ecosystems, and many of them are valuable forages in local rangelands. A few species are cultivated as perennial warm-season grasses, such as *P. notatum*, and *P. dilatatum*. There is an informal taxonomic group within *Paspalum* named Plicatula (Chase 1929), which contains the most interesting species considering the diversity of species and biotypes, and forage attributes. This group has approximately 30 species and most of them are tetraploid and apomictic (Ortiz et al. 2013). Direct selection from plant collections of several species of this group resulted in the release of forage cultivars along the years (Evers and Burson 2004).

The collection of a sexual diploid cytotype of *P. plicatulum* in northeastern Argentina, and its chromosome duplication resulted in the generation of a sexual self-incompatible tetraploid plant (Sartor et al. 2009). This sexual plant was successfully crossed with three accessions belonging to three species of Plicatula, i.e., *P. guenoarum* (Aguilera et al. 2011), *P. chaseanum* (Novo et al. 2013), and *P. oteroi* (Novo et al. 2016).

The genetic distance between parents appears to affect hybridization efficiency and hybrid performance in most plant species. Within a single species heterosis usually increases with increased genetic divergence between parents (Poehlman and Sleper 1995). However, sterility and poor hybrid performance are common in crosses involving different species. Thus, the genetic distances between species and accessions of Plicatula are expected to be related to the hybridization outcomes. However, it is supposed that the genetic distance between two species should have a critical point beyond which it becomes

a restriction for crossability or for the fertility of the hybrids if these are obtained.

The inheritance of apomixis was evaluated in crosses between *P. plicatulum* and *P. guenoarum*, and the trait was inherited as a simple Mendelian dominant factor (Aguilera et al. 2015). These results are in agreement with previous observations in *P. notatum* (Martínez et al. 2001), and also with other grass genera, such as *Brachiaria* and *Panicum* (Miles 2007). Since sexual hybrids are supposedly in a nulliplex condition for the apomixis locus, a sexual population could be created by polycrossing the sexual germplasm. If hybridization is possible between the sexual tetraploid genotype of *P. plicatulum* and different species and accessions of the Plicatula group, the generation of a broad-base sexual population might be possible.

The possibility of fixing superior hybrids in each hybridization cycle is what makes apomixis an interesting trait (Hanna and Bashaw 1987). Thus, the occurrence of heterosis in hybrids resulting from crosses between sexual and apomictic plants is essential to reach this goal. Previous research indicated that it is possible to obtain a proportion of the generated hybrids, from crosses between sexual and apomictic genotypes of *Paspalum notatum*, which are superior to their parents for several agronomic traits (Acuña et al. 2011; Zilli et al. 2015). The analysis of heterosis in the Plicatula group is expected to indicate the possibility of manipulating apomixis for fixing outstanding intra- and inter-specific hybrids.

The objectives of this research were to (i) cross several apomictic tetraploid species and accessions of the Plicatula group of *Paspalum* with sexual colchicine-induced tetraploid *P. plicatulum* as female parent, (ii) estimate the genetic distance between sexual and apomictic parents, (iii) evaluate the relationship between the genetic distance between parents and the crossability and fertility of the progeny, and (iv) analyze the occurrence of heterosis for agronomic traits in the most vigorous apomictic hybrids.

Materials and methods

Plant material, chromosome counting, and crosses

The original material for this research consisted of an induced sexual tetraploid genotype of *P. plicatulum*

(Sartor et al. 2009), and 22 accessions belonging to 12 different species of the Plicatula group of *Paspalum*, including *P. plicatulum* (Table 1). Chromosome numbers for new accessions were determined following the procedure used by Zilli et al. (2014). Crosses were made using the sexual plant as a female parent and the apomictic tetraploid accessions as male parents between March 2009 and January 2012. Crosses were made following the procedure previously described by Novo et al. (2013). Inflorescences from the female parent were immediately covered with glassine bags after pollination. After one month seed heads were threshed, and the total number of pollinated florets, and generated seeds were counted. Seed set was calculated by dividing the number of florets containing cariopses by the total number of pollinated florets and multiplying by 100. Germination was tested in seed trays using sterile soil at the beginning of the following spring. Crossability was calculated as the number of recovered hybrids per one hundred pollinated spikelets. Seedlings were transplanted to small pots in a greenhouse and then to a space-plant nursery in the field.

Analysis of hybrid origin, and fertility

Genomic DNA was extracted from leaves of the progeny and parents using the technique developed by Dellaporta et al. (1983). Random Amplified Polymorphism (RAPD) was used to determine the origin of the progeny as previously described by Novo et al. (2013). The amplification of a minimum of three markers specific of the male parent was considered for confirming the hybrid origin of the generated plants.

Hybrids transplanted into a space-plant nursery were used for evaluating their fertility based on seed set under open pollination. A minimum of 4 inflorescences of each hybrid were covered with glassine bags a day after all their spikelets completed flowering, and the inflorescences remained covered for 20 days before harvest. Only a sample of hybrids was randomly selected from each progeny for this purpose. The number of selected hybrids depended on availability of adequate flowering heads when the experiment was conducted. The mature seed heads were dried, threshed, and seeds were separated from empty spikelets by a blower and counted separately.

Genetic distances

Amplified fragment-length polymorphism markers (AFLP) were used to determine the genetic distance between a sexual genotype of *P. plicatulum* and 22 tetraploid apomictic genotypes (Table 1) according to the technique described by Vos et al. (1995). The AFLP procedure was undertaken following the manufacturer's instructions of the AFLP *Analysis System I* (*Invitrogen*, Carlsbad, USA). About 900 ng of genomic DNA were simultaneously digested with EcoRI (E) and MseI (M). The restricted genomic DNA fragments were ligated to EcoRI and MseI adapters, and constituted the template for further amplifications. Twenty primer combinations were screened (E31M31, E31M32, E31M33, E31M35, E31M42, E32M32, E32M33, E32M39, E33M32, E33M33, E34M35, E34M42, E35M32, E35M37, E35M42, E38M32, E38M39, E41M34, E41M38, E41M4) on the DNA of the two genotypes (GR19 and V&A1332), and the most informative combinations were selected. The pre amplifications and selective amplifications were performed as described by Aguilera et al. (2015). Amplification products were run in 5% polyacrylamide gels, stained with silver, and digitalized using HP Scanjet 4670 scanner (Hewlett-Packard).

Reproductive mode of hybrids and field evaluations

The mode of reproduction of hybrids was classified as sexual or apomictic by the flow cytometric seed screening (FCSS) method developed by Matzk et al. (2000), following the procedures previously described by Aguilera et al. (2015). For this analysis, seeds were harvested from each F₁ hybrids following open pollination. For the present study, only the most numerous families were analyzed, i.e., 4PT × *P. plicatulum* Hojs388; × *P. guenoarum* GR19, Azulao and Baio. Once the method of reproduction was known, the most vigorous apomictic hybrids of these four progenies were selected.

These selected apomictic hybrids and the respective apomictic parents were grown in a greenhouse and planted into a field located in Riachuelo (27°38'S, 58°44'W), Province of Corrientes, Argentina on October 2011. Both were planted on 1-m centers in a randomized complete block design with three

Table 1 Origin, and ploidy levels of species and accessions of the Plicatula group of *Paspalum*

Species	Accession	Origin	Chromosome numbers	
			2n	References
<i>P. chaseanum</i> Parodi	ST13894	98 km E of Boyuibe, Transchaco route, department of Cruz, Bolivia	40	Novo et al. (2013)
<i>P. compressifolium</i> Swallen	AK40811	Vila Velha, Paraná, Brazil	40	Honfi et al. (1990)
<i>P. guenoarum</i> Arechav	Azulao	Unknown locality of Southern Brazil, received from the Federal University of Rio Grande do Sul, Porto Alegre, Brazil	40	Hickenbick et al. (1987)
	Baio	Unknown locality of Southern Brazil, received from the Federal University of Rio Grande do Sul, Porto Alegre, Brazil	40	Hickenbick et al. (1987)
	BO107	Paraguay, received from Margot Forde Forage Germplasm Centre, New Zealand	40	This work
	GR19	Paraguay, cv Rojas, seed received from Zaiman experimental station of INTA, Misiones, Argentina	40	Aguilera et al. (2015)
	Q4108	Seed received from CIMBOC (Centro de Investigación y Mejoramiento Bovino Criollo), Luis Calvo, Chuquisaca, Bolivia	40	This work
<i>P. lenticulare</i> Kunth	V11739	Vicinity of Ilha Solteira dam, San Pablo, Brazil	40	This work
	BO190	Seed received from Margot Forde Forage Germplasm Centre, New Zealand, released as cv Bryan of <i>P. plicatum</i> .	40	This work
<i>P. macedoi</i> Swallen	TK2323	25 km S of Concepción, Santa Cruz, Bolivia	40	Norrmann et al. (1994)
<i>P. modestum</i> Mez	Hojs395	Km 1258, national route 12, Ituzaingó, Corrientes, Argentina	40	This work
<i>P. nicorae</i> Parodi	CPI27707	Santa María, Rio Grande do Sul, Brazil. Seed received from Tropical crops and forages collection Australia	40	This work
	PI508821	Arroyo Sauce, national route 14, S of Guleguaychú, Entre Ríos, Argentina. Seed received from Texas A&M University.	40	This work
<i>P. oteroi</i> Swallen	A&V1332	Fazenda Firme, Carumbá, Brazil.	40	Pozzobon and Valls (1987)
<i>P. palustre</i> Mez	BO110	13 km SSW of Ibarreta, Formosa, Argentina	40	Galdeano et al. (2016)
<i>P. plicatum</i> Michx.	4PT	Colchicine-induced tetraploide, Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste, Corrientes, Argentina	40	Sartor et al. (2009)
	Hojs388	Km 1066 of national route 12, Department of San Cosme, Argentina	40	Galdeano et al. (2016)
	ML5	Km 266, national route 5, Uruguay, seed received from PGG Wrightson seeds, Montevideo, Uruguay.	40	This work
	Q4087	Curuzú Cuatiá, Corrientes, Argentina. Variety with shining dark brown sterile lemma.	40	This work
	<i>P. rojasii</i> Hackel	AK40732	Vila Velha, Paraná, Brazil	40
<i>P. wrightii</i> Hitchc. and Chase	Q4158	104 km E of Corrientes, Argentina	40	This work
	U89	3.5 km N of the access to Tres Isletas, national route 95, Chaco, Argentina	40	This work
	U90	Campus Deodoro Roca, Universidad Nacional del Nordeste, Corrientes, Argentina	40	This work

replications. The experimental unit was composed of three individual clonal plants of each apomictic hybrid per block. A border row of remaining plants was planted around the plot. The soil type was classified as Argiudoll.

The initial growth was estimated on 15 January 2012 using a 1–5 scale, where 1 represented the plants exhibiting the lowest amount of above-ground growth, and 5 represented the plants with the highest amount of growth. Seed set on open pollination was determined on 13 February by harvesting two mature seed heads from two plants in each block. Seed heads were dried, threshed, and seeds were separated and counted. The amount of leaf growth was estimated after seed harvest on 8 April using the same visual scale described above. Plants were then cut to approximately 10 cm stubble height on 30 April. Regrowth was estimated during fall on 30 May, and winter on 18 July 2012 and 3 July 2013.

Forage yield was determined by cutting individual plants at 8 cm stubble height on 7 November 2012, 20 December 2012, and 5 March 2013. The fresh weight of the harvested material was recorded and a subsample was collected and dried at 60 °C for 48 h. The dry subsample was weighted (g) and the amount of harvested biomass was calculated. Frost tolerance was visually estimated three days after the first frost event of the season on 18 June 2012 and 27 August 2013, using a 1–5 scale, where 1 = the least frost resistant, and 5 = the most resistant plant. Cattle preference was estimated by allowing 50 cows to graze the plot area during 1 h on 3 September 2013. The estimation was carried out using a 1–5 visual scale, where 1 = the least preferred genotype, and 5 = the most preferred.

Statistical analyses

DNA amplification profiles obtained with AFLP molecular markers 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 (Balzarini and Di Rienzo 2004). The genetic distance among parents was estimated using the Jaccard's dissimilarity coefficient (Kosman and Leonard 2005).

Analysis of variance (ANOVA) and mean separations by the Tukey test were calculated for the agronomic data. Apomictic-parent heterosis values

were calculated as mean differences using the Tukey test for comparing mean differences between families or individual apomictic hybrids, and apomictic parents. The proportion of hybrids exhibiting positive or negative apomictic-parent heterosis was calculated for all traits.

Results

A total of 22 crosses between tetraploid sexual and apomictic genotypes were made from March 2009 to January 2012 (Table 2). Most crosses were inter-specific (19) involving 11 species, and a variable number of accessions in some species. Intra-specific crosses were also made including three different accessions of *P. plicatulum*. The number of pollinated florets was variable among crosses depending on the synchrony of flowering between female and male parents. The total number of pollinated spikelets was 9440 considering all inter- and intra-specific crosses. Seeds were obtained in 17 parental combinations, and the seed set (proportion of pollinated spikelets that formed caryopsis) varied among them between 1 and 51.5% (Table 2). The highest crossability was observed for the crosses where *P. plicatulum* Hojs388, and three accessions of *P. guenoarum* (Azulao, Baio, and GR19) were used as male parents. A total of 427 plants germinated out of 922 seeds; however, germination varied between 10.2 and 100% among crosses.

A progeny test was performed with RAPD markers to evaluate the actual origin of the obtained plants. The analysis was carried out using a sample of 95 plants, resulting from 12 different parental combinations. All analyzed plants had a minimum of 3 molecular markers that were present only in the apomictic parent indicating that they were hybrids (Table 3). The family with the largest number of plants (4PT × GR19) was previously analyzed using AFLP (Aguilera et al. 2015), and also all tested plants were determined to be hybrids. Thus, a large sample of 184 out of 427 plants from 13 different full-sib families was analyzed and confirmed the hybrid origin of the generated progeny. In addition, this result indicated that the sexual colchicine induced autotetraploid 4PT plant of *P. plicatulum* is completely self-sterile but cross-fertile.

The genetic distances among the sexual parent 4PT and each apomictic parent were determined

Table 2 Crosses between an induced sexual tetraploid genotype (*P. plicatum* 4PT) and a group of apomictic accessions of different species of *Plicatula*: genetic distance between parents, number of pollinated florets in each cross, germination

of the generated seed, crossability (proportion of generated plants considering the number of pollinated florets), and seed fertility under open pollination of the obtained hybrids

Crosses	Genetic distance	Pollinated florets no.	Seed set %	Hybrids no.	Germination %	Crossability %	Fertility of hybrids %
<i>P. plicatum</i> 4PT							
× <i>P. plicatum</i> Q4087	0.34	449	0.0	–	–	–	–
× <i>P. plicatum</i> ML5	0.35	360	3.6	13	100	3.6	40.1
× <i>P. rojasii</i> AK40732	0.37	816	2.8	17	48.6	2.1	10.4
× <i>P. guenoarum</i> BO107	0.38	94	6.4	4	66.7	4.2	15.0
× <i>P. guenoarum</i> GR19	0.38	1282	19.0	189	77.5	14.7	31.3
× <i>P. guenoarum</i> Q4108	0.38	647	1.7	2	18.2	0.3	22.1
× <i>P. guenoarum</i> Baio	0.39	367	19.6	59	81.9	16.1	17.0
× <i>P. macedoi</i> TK2323	0.40	96	0.0	–	–	–	–
× <i>P. chaseanum</i> ST13894	0.40	254	11.8	27	90.0	10.6	3.4
× <i>P. nicorae</i> CPI27707	0.40	992	1.1	3	27.3	0.3	31.3
× <i>P. guenoarum</i> Azulao	0.41	572	32.0	45	24.6	7.9	20.7
× <i>P. nicorae</i> PI508821	0.43	1124	13.1	15	10.2	1.3	21.2
× <i>P. plicatum</i> Hojs388	0.43	235	51.5	20	16.5	8.5	12.7
× <i>P. lenticulare</i> BO190	0.44	168	4.8	4	50.0	2.4	6.3
× <i>P. wrightii</i> U90	0.45	382	1.3	1	20.0	0.3	–
× <i>P. lenticulare</i> V11893	0.46	131	3.0	4	100	3.0	1.6
× <i>P. wrightii</i> Q4158	0.46	271	0.0	–	–	–	–
× <i>P. compressifolium</i> AK40811	0.47	877	1.0	2	22.2	0.2	16.9
× <i>P. wrightii</i> U89	0.47	137	0.0	–	–	–	–
× <i>P. modestum</i> Hojs395	0.50	569	0.0	–	–	–	–
× <i>P. palustre</i> BO110	0.52	115	0.0	–	–	–	–
× <i>P. oteroi</i> A&V1332	0.53	479	4.8	22	95.7	4.6	27.8

using AFLPs. Seven primers combination were selected, a total of 200 loci were amplified, and all of them were polymorphic. The genetic distances varied from 0.34 for the parents 4PT and Q4087 of one intraspecific cross within *P. plicatum*, to 0.53 for *P. plicatum* and *P. oteroi* parents of the corresponding interspecific crosses (Table 2).

Seed fertility (proportion of spikelets that formed caryopsis) was determined in a random sample of hybrids for each progeny based on the seed set under open pollination (Table 2). Mean seed fertility varied between 1.6% for the cross between *P. plicatum* and *P. lenticulare* V11893, and 40.1% for the intraspecific cross *P. plicatum* 4PT × ML5. There was not a correlation between genetic distances and seed

fertility or crossability. There were intraspecific crosses with low genetic distances between parents that did not hybridize (*P. plicatum*, 4PT × Q4087, crossability = 0), and another (*P. plicatum*, 4PT × ML5), that resulted in a crossability of 3.6%, and the resulted hybrids had a seed fertility of 40.1% (Table 2). No hybrids were obtained from some interspecific crosses with genetically distant parents, such as the cross where *P. palustre* BO110, *P. modestum* Hojs395 or *P. wrightii* U89 were the male parents. However, some distant interspecific crosses resulted in fairly fertile hybrids, such as *P. plicatum* 4PT × *P. oteroi* A&V1332 (Table 2).

The mode of reproduction of the four most numerous families was determined using flow

Table 3 Analysis of hybrid origin based on RAPDs

Crosses	Analyzed F ₁ no.	Selected primers no.	Amplified parental markers no.	Male-parent-specific marker no.
<i>P. plicatulum</i> 4PT				
× <i>P. chaseanum</i> ST13894	24	3	18	7
× <i>P. compressifolium</i> AK40811	1	2	9	4
× <i>P. guenoarum</i> BO107	4	3	19	5
× <i>P. guenoarum</i> Q4108	1	2	19	3
× <i>P. lenticulare</i> BO190	4	2	15	4
× <i>P. nicorae</i> CPI27707	1	3	19	4
× <i>P. nicorae</i> PI508821	15	4	23	7
× <i>P. oteroi</i> A&V1332	12	4	24	10
× <i>P. plicatulum</i> Hojs388	13	2	19	11
× <i>P. plicatulum</i> ML5	7	2	12	3
× <i>P. rojasii</i> AK40732	12	3	15	6
× <i>P. lenticulare</i> V11893	1	2	8	3

Number of analyzed plants from each cross, number of primers used for each progeny, number of amplified specific markers from parents, and number of amplified male-specific marker

Table 4 Segregation for mode of reproduction in four families resulting from crossing sexual and apomictic genotypes of the *Plicatula* group of *Paspalum*

Crosses	Analyzed F ₁ no.	Sexual no.	Apomictic no.	Ratio sex./apo.	Selected hybrids no.
<i>P. plicatulum</i> 4PT					
× <i>P. guenoarum</i> Azulao	45	22	23	1.0:1	20
× <i>P. guenoarum</i> Baio	56	27	29	0.9:1	25
× <i>P. guenoarum</i> GR19 ^a	182	112	70	1.6:1	36
× <i>P. plicatulum</i> Hojs388	18	7	11	0.6:1	7

The number of apomictic hybrids evaluated in the field is also included

^a From Aguilera et al. (2015)

cytometric seed screening (Table 4). The ratio between sexual and apomictic hybrids varied between 0.6:1 and 1.6:1. Seed from the most vigorous apomictic hybrids were sown in a greenhouse, and the resulting plants were planted into the field. A total of 88 hybrids were planted together with the apomictic parents for comparing the agronomic performance of both generations and the possible occurrence of heterosis. The sexual parent was not included in this field experiment because of its reduced vigor.

Significant differences were observed among hybrids and families for all the evaluated traits. No positive and significant heterosis was observed for

initial growth, regrowth after seed harvest, seed set, fall or winter regrowth (Table 5). Most hybrids were not significantly different in comparison with their apomictic parents. A low proportion of the evaluated hybrids exhibited negative heterosis for these agronomic traits. Between 0 and 12% of the hybrids in each family were significantly inferior in comparison with their apomictic parents for initial growth (Fig. 1). Similar values were observed for regrowth after seed harvest (0–25%), seed set (0–5%), fall regrowth (0–28%), 2012 winter regrowth (0–25%), and 2013 winter regrowth (0–14%) (Fig. 1).

Differences between apomictic parents and their apomictic progeny were also observed for forage

Table 5 Apomictic-parent heterosis for initial growth, regrowth after seed harvest, and seasonal regrowth in apomictic hybrids from four different tetraploid families of the Plicatula group of *Paspalum*

Male parent/ Family	Initial Growth	HPH	RSH	HPH	Seed set (%)	HPH	Fall regrowth	HPH	Winter regrowth 2012	HPH	Winter regrowth 2013	HPH
Azulao	4		4.67		32.49		5		5		4.33	
4PT × Azulao	2.98	-1.02	3.8	-0.87	19.67	-12.82	3.2	-1.8	3.67	-1.33	3.28	-1.05
Hojs388	2.33		3		20.95		2.33		2.33		3.33	
4PT × Hojs388	1.95	-0.38	2.9	-0.1	10.63	-10.32	1.86	-0.47	2.1	-0.23	2.63	-0.7
Baio	4		4.33		18.08		4.33		3.67		4.67	
4PT × Baio	3.45	-0.55	3.69	-0.64	20.87	2.79	3.63	-0.7	4	0.33	3.91	-0.76
GR19	4.6		5		30.82		3.8		3.8		4	
4PT × GR19	3.59	-1.01	3.81	-1.19	23.71	-7.11	3.79	-0.01	4.18	0.38	4.24	0.24
MSD 0.05	1.01		0.80		12.48		0.96		0.94		0.88	

HPH high-parent-heterosis, RSH regrowth after seed harvest

Fig. 1 Proportions of apomictic hybrids from the Plicatula group of *Paspalum* exhibiting significant apomictic-parent heterosis for six agronomic traits. Hybrids that were not statistically different from the respective comparison are not represented in the bar graph

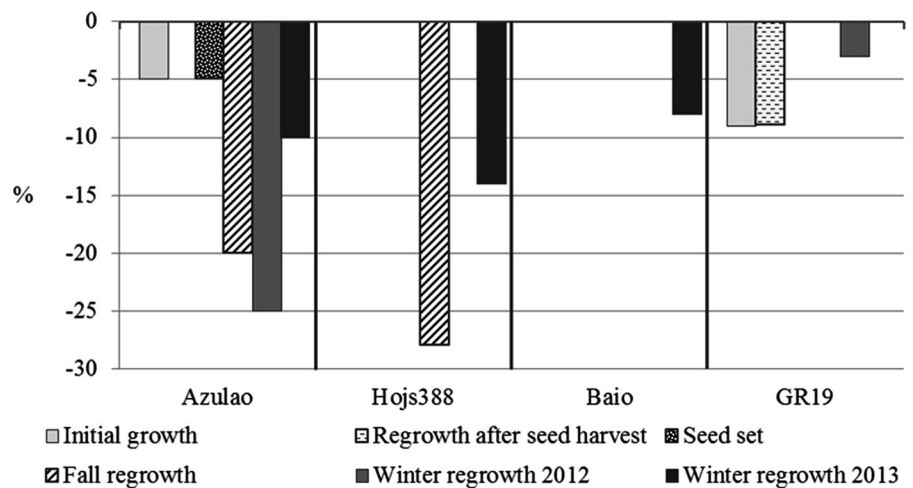
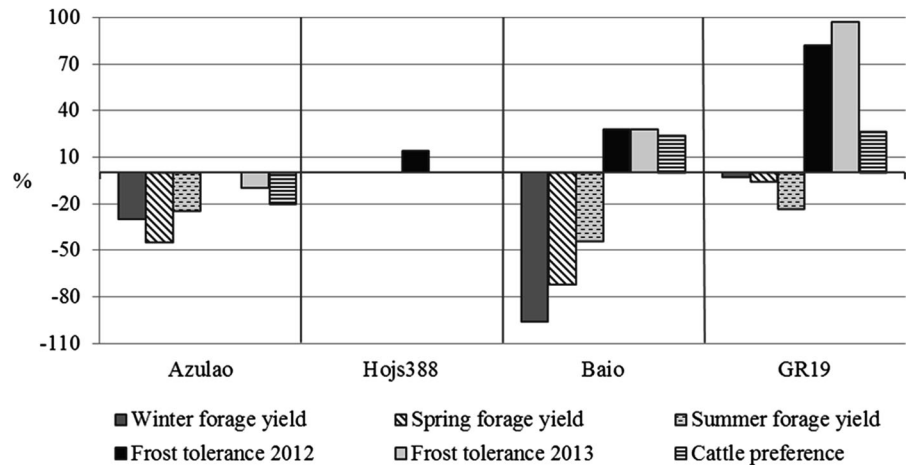


Table 6 Apomictic-parent heterosis for forage yield, frost tolerance and cattle preference in apomictic hybrids from four different tetraploid families of the Plicatula group of *Paspalum*

Male parent/family	Forage yield (g pl ⁻¹)						Frost tolerance 2012	HPH	Frost tolerance 2013	HPH	Cattle preference	HPH
	Winter	HPH	Spring	HPH	Sumer	HPH						
Azulao	222.57		355.96		774.94		3.33		3		4.33	
4PT × Azulao	123.89	-98.68	196.78	-159.18	394.18	-380.76	3.52	0.19	2.32	-0.68	2.85	-1.48
Hojs388	48.79		384.07		276.03		2		1.67		1	
4PT × Hojs388	28.17	-20.62	51.02	-333.05	162.88	-113.15	3.38	1.38	1.41	-0.26	1.35	0.35
Baio	408.02		544.46		1039.79		2		1.33		1.33	
4PT × Baio	147	-261.02	238.81	-305.65	604.94	-434.85	2.92	0.92	3.01	1.68	2.93	1.6
GR19	240.1		374.39		899.23		2		1		1.2	
4PT × GR19	155.91	-84.19	220.57	-153.82	504.36	-394.87	3.03	1.03	3.8	2.8	3.27	2.07
MSD 0.05	71.87		85.50		171.58		0.63		1.06		1.29	

HPH high-parent heterosis

Fig. 2 Proportions of apomictic hybrids from the Plicatula group of *Paspalum* exhibiting significant apomictic-parent heterosis for six agronomic traits. Hybrids that were not statistically different from the respective comparison are not represented in the bar graph



yield during the evaluated seasons (Table 6). Hybrids were inferior or not different from their apomictic parents. The proportion of hybrids exhibiting significant negative heterosis varied among families for each season (Fig. 2). It varied from 30 to 96% for winter, from 30 to 75% for spring and from 25 to 100% for summer.

Positive heterosis was observed for frost tolerance in 2012 and three out of the four evaluated families were significantly more tolerant than their apomictic parents (Table 6). The proportion of hybrids that were superior to their parents varied between 14 and 87% (Fig. 2). Similar results were found for frost tolerance during winter 2013, and two out of the four families were significantly superior in comparison to the apomictic parent (Table 6). These two families outperformed the male parents in both years (Table 6; Figs. 1, 2). The proportion of hybrids that exhibited positive heterosis varied from 28 to 100% (Fig. 2).

Positive heterosis was also observed for cattle preference, and 3 out of 4 families were superior to the apomictic parents (Table 6). The proportion of hybrids that were superior varied between 24 and 81% among families (Fig. 2).

Discussion

The release of the variability contained in the apomictic germplasm of the group Plicatula of *Paspalum* is essential for the genetic improvement of several species with high forage value. In this research we attempted to hybridize a new induced sexual

tetraploid genotype of *P. plicatulum* with 22 apomictic accessions belonging to 12 species of this group, including three accessions of wild apomictic tetraploid *P. plicatulum*. The observed variation for the efficiency of hybridization was expected, considering the diversity of species and genotypes collected from a vast geographical region. Unexpectedly, hybrids were obtained from 77% of the attempted crosses. The most successful crosses involved apomictic accessions belonging to *P. guenoarum*, *P. plicatulum*, *P. chaseanum*, *P. oteroi*, *P. rojasii*, *P. lenticulare*, and *P. nicorae*. The hybridization procedure was particularly efficient when accessions of *P. guenoarum* were used as male parents. Some of the generated hybrids were used for agronomic evaluations considering their potential as forages, and they could be utilized to analyze the relationship between the species and the evolutionary patterns that resulted in this taxonomic group.

The hybridization efficiency and seed fertility observed in the hybrids involving most accessions of *P. guenoarum* was in agreement with the close cytogenetic relationship observed previously between these two species. Aguilera et al. (2011) showed that tetraploid *P. plicatulum* × *P. guenoarum* hybrids had quite similar bivalent and quadrivalent chromosome associations to those observed at meiosis in their parents, indicated that both species are autotetraploids and share genomes with an important degree of homology. In this work, we were able to evaluate four accessions of *P. guenoarum* with contrasting morphological characteristics and collected from different geographical regions, and the results indicated that from all crosses fertile hybrids were obtained.

The results also confirmed that the induced sexual tetraploid plant used as female parent behaved as self-incompatible as it was previously reported by Sartor et al. (2009), Aguilera et al. (2011), Novo et al. (2013, 2016) and allowed us to accomplish an efficient hybridization procedure. No plant formed by self-pollination was recovered either in the present study considering that hybrids were obtained from 12 different crosses involving eight species despite that in all cases the plant 4PT of *P. plicatulum* was crossed without previous emasculation. However, chromosome duplication should be attempted with other diploid species of Plicatula with the objective of hybridizing other species and biotypes. Although sexual diploid genotypes were reported for *P. chaseanum*, *P. compressifolium*, *P. modestum*, *P. lenticulare*, *P. palustre*, and *P. wrightii* (Ortiz et al. 2013), the creation of sexual tetraploid genotypes had proven to be a difficult task in *Paspalum*.

The genetic distances observed between accessions and species of the group indicate that the taxonomic classifications in the Plicatula group are complex and in some cases should be reconsidered. The lack of a correlation between the genetic distances, crossability, and fertility of the generated hybrids might be an additional indication of this idea. Furthermore, combinations of genetically distant parents resulted in fairly fertile hybrids, such as the cross where *P. oteroi* was the male parent.

When the chromosome associations were evaluated, the mean number of bivalents and quadrivalents was similar between apomictic parents and hybrids in the two of the most successful crosses between medium to closely related parents, i.e., *P. plicatulum* × GR19 *P. guenoarum* (Aguilera et al. 2011) and *P. plicatulum* × *P. chaseanum* (Novo et al. 2013). However, a fairly regular meiosis was observed by Novo et al. (2016) in hybrids from *P. plicatulum* × *P. oteroi* crosses, with most chromosomes forming bivalents, and with several hybrids having a higher seed set than either parent. This meiotic behavior might be related with the good degree of fertility observed for crosses between genetically fairly distant parents that showed low crossability as is the case of hybrids between *P. plicatulum* × *P. compressifolium*. Further research is needed to evaluate the chromosome associations present in the rest of the hybrids, and genomic relationships among the parental species.

The inefficiency of the 4PT × Q4087 *P. plicatulum* interspecific cross deserves special consideration regarding taxonomic affinities. The apomictic genotype Q4087 belongs to a rare morphological variant of the species. As in most *Paspalum* species, the spikelets of typical *P. plicatulum* are plano-convex with the sterile lemma and the glume at the plain and the convex sides, respectively and both thin in texture and olivaceous to grayish in color. The general morphology of genotype Q4087 fits the description of the species but it has biconvex spikelets due to a convex, dark brown and indurate sterile lemma. This phenotype was described as a different botanical variety: *P. plicatulum* var. *intumescens* Doell. However, Chase (1929) in her monograph of the genus placed the Döll's variety into the synonymy of *P. plicatulum* Michx. The close genetic distance that we observed between Q4087 and other accessions of *P. plicatulum* is in agreement with this criterion. However, the inefficiency of our cross may indicate that Q4087 was prevented genetically from hybridizing the conspecific 4PT genotype.

A few crosses resulted in a large number of apomictic hybrids that were evaluated as individual plants under field conditions. The high diversity observed for traits of agronomic importance indicated that the objective of releasing the diversity locked in the apomictic parents was accomplished. Also, this high level of diversity supports the possibility of genetic improvement of *P. plicatulum* and *P. guenoarum*. All previous attempts for breeding these species were based on the evaluation of apomictic biotypes of each species (Evers and Burson 2004).

Positive apomictic-parent heterosis was observed for frost tolerance in two consecutive years, and for cattle preference. Although no positive heterosis was observed for the rest of the evaluated traits, most hybrids were not significantly inferior to the apomictic parents. The results indicate that the occurrence of heterosis is highly dependent of the analyzed trait. The proportion of hybrids exhibiting heterosis is also related to the combination of parents used. The induced sexual tetraploid used as female parent for all crosses has a very low vigor and it hardly survive under field conditions. Thus, future crosses using the new sexual hybrids as female parents may result in more productive hybrids. The results are in agreement with previous reports that resulted from the evaluation of a large number of apomictic hybrids of

P. notatum (Zilli et al. 2015), where heterosis was also mainly related with the trait of interest and the combination of parents used. In addition, positive heterosis for frost tolerance is a desirable character since all these species are primarily tropical or subtropical, and this trait may allow plant improvement for cultivation in more temperate regions.

In this research we were able to identify hybrids that combined superior cold tolerance and cattle preference, with high levels of seed fertility and growing capacity (not different from their apomictic parents). They have the potential to increase the extent of the regular growing period of warm-season grasses when cultivated in the subtropics, and also increase forage intake. However, these hybrids should now be included in more advanced phase of a forage evaluation program to analyze these initial observations.

In conclusion, a novel induced sexual genotype allowed for the release of the diversity contained in many apomictic species and accessions of the Plicatula group of *Paspalum*. The efficiency of hybridization and the fertility of the generated progeny were dependent on the male parent involved, but they were independent of the genetic distances between the sexual and apomictic parents. The generation of apomictic hybrids exhibiting heterosis for some cold tolerance and cattle preference was possible for crosses between *P. plicatum* and *P. guenoarum*. These hybrids will be included in advanced phases of forage evaluation aiming to develop a new apomictic cultivar.

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