CSIRO PUBLISHING

Australian Journal of Botany, 2013, **61**, 538–543
http://dx.doi.org/10.1071/BT13194

An apomictic tetraploid *Paspalum chaseanum* cytotype and its cytogenetic relationship with *P. plicatulum* (Poaceae): taxonomic and genetic implications

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Abstract. *Paspalum chaseanum* Parodi (Poaceae) is a rare species seldom found in the vast phytogeographic Chaco region of South America. It occurs in some localities as a diploid with 20 somatic chromosomes, reproduces sexually and is self-incompatible. A tetraploid cytotype was recently collected in this geographic region. This accession was determined to reproduce of aposporous apomixis and was crossed, as pollen donor, onto a sexual autotetraploid plant of *P. plicatulum* Michx. The meiotic chromosome pairing behaviour of both parents and their hybrids was primarily as bivalents and quadrivalents, indicating that tetraploid *P. chaseanum* is likely to have an autoploid origin, and that both species share basically the same genome. Although some controversies exist regarding the subgeneric taxonomic classification of *P. chaseanum*, these results support its inclusion in the informal Plicatula group of *Paspalum*. The *P. plicatulum* × *P. chaseanum* hybrids segregated for apomixis. The amount of seed set in some hybrids (up to 17%) and the presence of sexual as well as facultative apomictic individuals in the progeny suggest that gene transfer through hybridisation is a feasible tool in genetic-improvement programs concerning these forage grass species.

Received 23 May 2013, accepted 3 November 2013, published online 11 February 2014

Introduction

Paspalum is a large, polymorphic genus with a complex and wide array of geographic, morphological and taxonomic lineages (Giussani *et al*. 2009). The genus also exhibits a wide range of ploidy levels, genetic systems and modes of reproduction. Apomixis is the prevalent method of reproduction in the genus *Paspalum*. Most polyploids are aposporous apomicts and many of them are multiploid species, i.e. species that include different cytotypes from diploids to higher ploidy levels. Diploids reproduce sexually, whereas co-specific polyploids are apomictic (Quarin 1992; Ortiz *et al*. 2013).

Most *Paspalum* species are native to the Americas and several produce good-quality forage. A few species are widely used for forage. Existing commercial cultivars have been selected from natural germplasm collected mainly in South America. Because of the predominance of apomictic reproduction in many of these forage species, the selection of outstanding apomictic ecotypes has been the method of plant improvement used in breeding programs. The genetic stability of the cultivars is a consequence of their apomictic method of reproduction. Breeding apomictic polyploids through controlled crosses and subsequent selection requires the acquisition of sexual polyploid plants that can be used as the maternal parent and will cross with naturally occurring apomictic polyploid ecotypes. Doubling the chromosomes of diploid cytotypes using colchicine is a feasible way to produce sexual autotetraploid germplasm that can be crossed with naturally occurring co-specific tetraploid cytotypes (Forbes and Burton 1961).

Different infrageneric taxonomic categories based mainly on morphological characteristics have been proposed by several authors, and a molecular-based phylogeny comprising the whole genus is still wanted, even though partial phylogenetic studies are available (Giussani *et al*. 2009; Rua *et al*. 2010). We follow the infrageneric categories recognised by Zuloaga and Morrone (2005) that consist of three subgenera: *Anachyris*, *Ceresia* and *Paspalum*. These authors also maintained a list of 28 informal taxonomic groups belonging to the subgenus *Paspalum* as they had been established by Chase (1929).

One of the most important informal categories is the Plicatula group that consists of ~30 species, most being productive components of native pastures in South American grasslands. The name of the group originates from *P. plicatulum* Michx., the species that was first described for this group. The placement of *P. chaseanum* Parodi in the Plicatula group has been controversial. *Paspalum chaseanum* lacks the typical wrinkles on the sterile lemma that are characteristic of the spikelets in the Plicatula group (*plicatulum* in Latin meaning wrinkled).

We attempted interspecific crosses between tetraploid *P. plicatulum* and tetraploid *P. chaseanum* to (1) investigate the genomic relationship between these two species by observing the meiotic chromosome associations in their hybrids and

(2) determine the feasibility of transferring genes between these two species for future genetic improvement.

Materials and methods

A sexual reproducing autotetraploid plant of *P. plicatulum*, identified as 4PT, was used as the maternal parent, whereas *P. chaseanum*, accession ST13894, was the pollen donor in the interspecific crosses. The 4PT accession was obtained from cuttings of an induced autotetraploid plant (Sartor *et al*. 2009). The accession ST13894 was collected by Carlos Saravia-Toledo at 98 km east of Boyuibe, Transchaco route, Department of Santa Cruz, province of Cordillera, Bolivia, and cultivated from seeds at Corrientes, Argentina.

Because 4PT is a self-incompatible plant, crosses were made without emasculating the maternal parent. In the afternoon, before the onset of blooming the following morning, a selected inflorescence of 4PT was enclosed in a glassine bag. The next morning, the glassine bag was removed soon after anthesis had occurred and the freshly exposed pistils were dusted with pollen from ST13894. The inflorescence was enclosed again in a glassine bag and pollination was repeated every morning until all of the florets on the inflorescence had completed flowering. One month later, the inflorescence was harvested, spikelets were threshed, cleaned and the ones filled with caryopsis were separated from the empty ones. Seeds were germinated the following spring in a tray containing sterilised soil; seedlings were planted into small pots in the greenhouse and later transplanted into a space-planted field nursery.

The chromosome number of *P. chaseanum* was determined using root tips. The roots tips were collected, pre-treated in a saturated solution of 1-bromonaphthalene for 2 h and immediately hydrolysed with 5 N HCl for 10 min at a room temperature without previous fixative treatment. Root tips were stained with Feulgen's reagent and smears were mounted in aceto-carmine and observed with a transmitted-light microscope.

Meiotic chromosome associations were observed for *P. chaseanum* and some of the hybrids in pollen mother cells (PMC). Young inflorescences were collected, fixed in a 5 : 1 solution of 100% ethanol : lactic acid and stored in 70% ethanol at 4−7°C. Anthers were dissected from florets and squashed in a drop of aceto-carmine under a cover slide. The PMCs were analysed with a transmitted-light microscope.

Genomic DNA (DNA) was extracted from fresh leaves of parental plants and their offspring according to Dellaporta *et al*. (1983). The hybrid origin of the offspring was confirmed by using random amplified polymorphism DNA (RAPD). Decamers acquired from the University of British Columbia (Vancouver, Canada) were used for polymerase chain reaction (PCR) amplifications as described in Aguilera *et al*. (2011). Electrophoresis was performed using 2% agarose gel. Both parents were analysed to find out bands that were specific of the paternal parent. Consequently, the presence of these specific bands in the profile of any plant was an indicator of its hybrid origin.

The method of reproduction of the paternal parent *P. chaseanum* and its hybrids with 4PT *P. plicatulum* was determined by two different procedures. The relative DNA content ratio between embryo and endosperm nuclei of mature seeds was determined by flow cytometry, according the technique described by Matzk *et al*. (2000). Plants that produced seeds with a 2 : 3 DNA content ratio for the embryo and the endosperm were considered to reproduce sexually. Plants that produced some seeds with a 2 : 5 embryo : endosperm ratio were considered to be apomictics or facultative apomictics. For the second procedure, the ovaries were cleared and observed with differential interference contrast microscopy (DIC). Inflorescences at anthesis were fixed for 24 h in 70% ethanol : 37% formaldehyde : glacial acetic acid (FAA, 18 : 1 : 1). The pistils were then dissected and cleared according the procedure outlined by Young *et al*. (1979). Individual plants with mature ovules bearing a single meiotic embryo sac (MES), which always had an egg cell, two synergids near the micropile, a two-nucleated central cell, and a cluster of antipodal cells in the chalazal end of the sac, were considered to reproduce sexually. Plants that contained ovules with aposporous embryo sac/s (AES) of the *Paspalum*-type, MES + AES, or some ovules with MES and some other with AES, were considered to reproduce apomictically, being either obligate or facultative, depending on whether the plant developed exclusively AES, or MES plus AES in its ovules, respectively. The so-called *Paspalum*-type of aposporous embryo sac was often organised as an egg cell, a binucleate central cell, and eventually one or two synergids near the egg cell. Antipodal cells were never present in this type of embryo sac.

Mature panicles were harvested to assess the fertility of *P. chaseanum* and some of its hybrids with *P. plicatulum*. Previously, panicles were allowed to complete flowering and then covered with glassine bags until harvest, to prevent seed loss from shattering. Additionally, some *P. chaseanum* inflorescences were covered before anthesis to determine the ability of the plants to set seed under self-pollinated condition. Harvested panicles were threshed and all spikelets were counted. The proportion of spikelets with caryopses indicated the seed-set performance.

Table 1. Meiotic chromosome configurations at diakinesis and metaphase I of *Paspalum plicatulum* (4PT), *P. chaseanum* (ST13894) and three of their interspecific hybrids

I, univalent; II, bivalent; III, trivalent; and IV, quadrivalent

Species or hybrid	No. of plants	No. of PMC	Range				Average per cell			
			I	II	III	IV	I	II	III	IV
P. plicatulum (4PT)[A]	1	53	0–1	8–18	0–1	1–7	0.1	14.2	0.1	2.8
P. chaseanum (ST13894)	1	23	0–7	9–18	0–3	0–5	2.4	14.0	0.5	2.0
4PT × ST13894 hybrids	3	60	0–6	7–20	0–2	0–6	1.9	13.6	0.3	2.5

[A]From Sartor *et al*. (2009).

Results

Paspalum chaseanum

The number of chromosomes in root-tip cells of the *P. chaseanum* accession indicated it was a tetraploid with $2n = 4x = 40$ chromosomes. The meiotic chromosome-pairing behaviour was analysed in 23 PMCs during diakinesis and metaphase I. Univalent, bivalent, trivalent and quadrivalent associations were observed; however, most associated as bivalents and quadrivalents (Table 1, Fig. 1*a*). The mean number of quadrivalents per PMC was two and up to five quadrivalents were observed in some PMC.

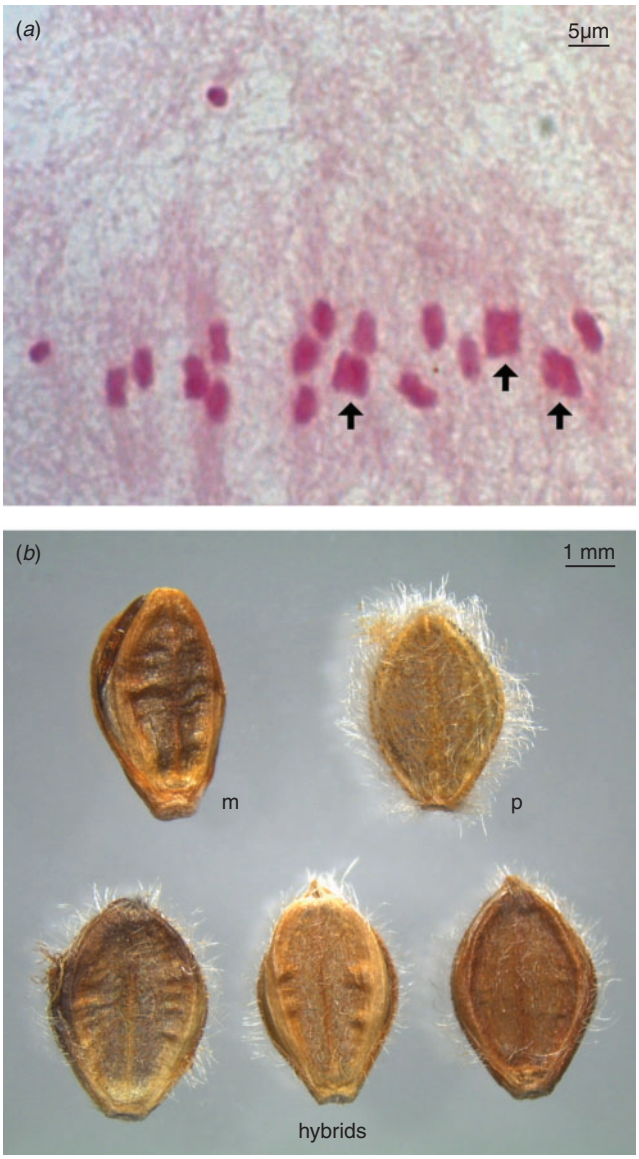

Fig. 1. Chromosome pairing in an apomictic tetraploid cytotype of *Paspalum chaseanum* ($2n = 4x = 40$) and the appearance of spikelets of *P. plicatulum*, *P. chaseanum*, and their F_1 hybrids. (*a*) Meiotic chromosome associations in *P. chaseanum*, showing two univalents, 13 bivalents and three quadrivalents (arrows). (*b*) Spikelets of the parental species and three F_1 hybrids; *P. plicatulum*, maternal parent (m), showing glabrous spikelet with wrinkled sterile lemma; *P. chaseanum*, paternal parent (p), with pubescent spikelet and smooth, sterile lemma. Variable degrees of pubescence and wrinkles are shown on the spikelets of three F_1 interspecific hybrids.

Fifty pistils of *P. chaseanum* were cleared to observe the mature embryo sacs. Most ovules contained a sac with an egg cell and two polar nuclei. Antipodals and synergids were absent, although one synergid was occasionally observed beside the egg cell. Less than 20% of the ovules had two embryo sacs similar in structure, although one sac was usually smaller than the normal one or it had deteriorated. The structure of these sacs suggested that they are aposporous sacs.

The relative DNA content in the embryo and endosperm tissues was determined for 15 mature seeds by single-seed analysis using flow cytometry. All seeds had a 2 : 5 ratio in the DNA content of their embryo : endosperm tissues. This agrees with the typical embryo : endosperm DNA content ratio when the seed embryo is formed following parthenogenesis in an aposporous embryo sac ($2n + 0$) and pseudogamous development of the endosperm after syngamy of two unreduced polar nuclei ($2n$ each) and a reduced sperm nucleus (n). The flow-cytometry single-seed analysis confirmed that the tetraploid cytotype of *P. chaseanum* reproduced by apomixis.

Seed set was 38% when inflorescences were enclosed in glassine bags to ensure self-pollination and 58% of the open-pollinated spikelets produced caryopses. These data showed that the apomictic tetraploid *P. chaseanum* accession set seed through autogamy, although it was more efficient under open-pollinated condition.

Hybrids

Pistils of 254 florets of sexual self-sterile autotetraploid *P. plicatulum* were dusted with pollen of apomictic tetraploid *P. chaseanum*. Thirty spikelets developed caryopsis and 27 seedlings were recovered; 24 survived and were transplanted into the field. The crossability between the two species was 9.4% ($24/254 \times 100$).

Although the F_1 hybrids were morphologically variable, the majority of them were more vigorous than was either parent. Both parents have plane-convex spikelets of similar sizes and shapes. The main difference was the transverse wrinkles on the sterile lemma of the glabrous *P. plicatulum* spikelets, in contrast to the smooth surface of sterile lemma of the densely pubescent spikelets of *P. chaseanum*. The 24 F_1 hybrids inherited the wrinkled trait from the maternal parent and the pubescence on the sterile lemma and second glume from the paternal parent. With regard to these quantitative attributes, the hybrids were intermediate between both parents. All exhibited less wrinkled sterile lemmas than in the maternal parent and less pubescence on the glumes and sterile lemmas than in the paternal parent (Fig. 1*b*). The presence of pubescent spikelets indicates that all 24 plants recovered are hybrids.

All the purported hybrids were analysed with RAPD markers to confirm their hybrid origin. Thirty eight decamers were screened to identify polymorphisms between both parents. Twelve did not produce amplification products. The remaining decamers produced satisfactory amplification profiles, but only three (BC421, BC756 and BC767) were selected for progeny analysis. These selected primers had amplified clear bands and were specific to the paternal parent. All of the purported hybrids

amplified some paternal-specific bands, indicating that all 24 individuals were hybrids (Fig. 2).

Meiotic chromosome behaviour was analysed in Hybrids 1, 23 and 25 (Table 1). The majority of the chromosomes in these hybrids associated primarily as bivalents and quadrivalents during diakinesis and metaphase I; however, some chromosomes remained unpaired (univalents) or rarely formed trivalents. The mean chromosome associations observed in the three hybrids were 1.9 univalents, 13.6 bivalents, 0.3 trivalents and 2.5 quadrivalents per PMCs (Table1). A maximum of six quadrivalents were observed in some PMCs.

Fertility (seed set) and mode of reproduction were determined for 14 of the 24 hybrids. Five hybrids did not produce any seed. Fertility in the remaining nine hybrids was variable, ranging from 0.4 to 17.2% (Table 2).

The method of reproduction was determined by microscopically observing cleared mature ovules and by determining the relative DNA content in the nuclei of embryo and endosperm tissue in individual seeds (Table 2). Observing the cleared ovaries provided more information regarding the actual developmental processes occurring in the ovule. The ovary-clearing technique was valuable for the analysis of the 14 selected hybrids, whereas flow-cytometry seed screening (FCSS) was exclusively applicable to those hybrids that produced seed. Equivalent results were observed with clearing technique and FCSS, except for Hybrids 6 and 9, which were determined to be facultative apomicts by embryological studies. However, FCSS indicated that all the seeds analysed for these two hybrids developed by sexual pathways. Because of the high levels of sexuality revealed by clearing technique (63.3 and 76.2%), and the shortage of seeds analysed by FCSS in both facultative apomictic Hybrids 6 and 9 (25 and 23 seeds, respectively), it is possible that we missed some potential seed formed through apomictic pathways.

Discussion

All of the previously collected *P. chaseanum* accessions that have been studied cytologically were sexual, self-incompatible diploids (Espinoza and Quarin 1997). However, accession ST13894 is an important tetraploid ($2n = 4x = 40$) that probably originated by autoploidy. Its meiotic chromosome-pairing

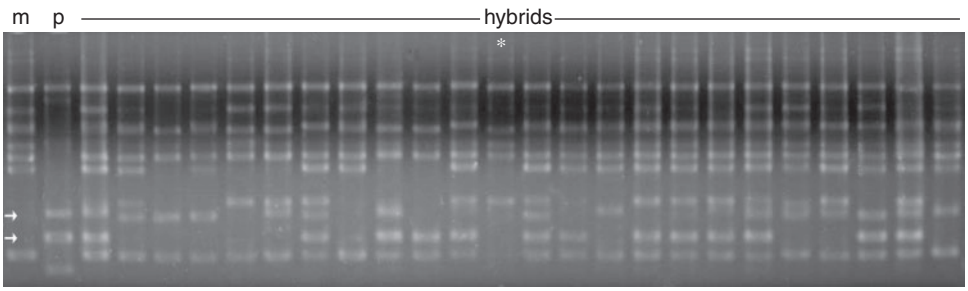

Fig. 2. Random amplified polymorphism DNA (RAPD) fingerprints produced by primer BC756 in parental species and 24 interspecific hybrids; *P. plicatulum*, maternal parent (m); *P. chaseanum*, paternal parent (p). Arrows show two paternal-specific bands. In combination, both bands demonstrate the hybrid origin of 23 of 24 purported hybrids. The hybrid origin of the remaining individual (*) was verified by another primer.

Table 2. Reproductive method and seed-set performance of 14 *Paspalum plicatulum* × *P. chaseanum* interspecific hybrids

The method of reproduction was determined by embryological observations (structural type of mature embryo sacs; clearing technique) and by the embryo + (endosperm) relative DNA contents (2C-values) of their seeds determined by flow-cytometry seed screening (FCSS). Abnormal, proportion of ovules lacking embryo sac or with a deteriorated sac; Fap, facultative apomictic hybrid; Seed set, percentage of spikelets that developed caryopses; Sex, sexually reproducing hybrid

Identity of hybrid	Type of embryo sacs observed by clearing technique in mature ovaries				Embryo + (endosperm) DNA content (C-values)			Reproductive mode according to		Seed set
	Number of cleared ovaries	Sexual (%)	Aposporous (%)	Abnormal (%)	Seed sample (no.)	2C+(3C) (%)	2C+(5C) (%)	Embryology	FCSS	
01	23	78.3	0.0	21.7	20	100.0	0.0	Sex	Sex	17.2
02	27	96.3	0.0	3.7	20	100.0	0.0	Sex	Sex	7.1
03	26	76.9	0.0	23.1	20	100.0	0.0	Sex	Sex	3.8
04	27	51.9	0.0	48.1	20	100.0	0.0	Sex	Sex	3.3
05	29	31.0	38.0	31.0	–	–	–	Fap	–	0.0
06	30	63.3	16.7	20.0	25	100.0	0.0	Fap	Sex	3.3
07	21	57.1	0.0	42.9	–	–	–	Sex	–	0.0
09	21	76.2	14.3	9.5	23	100.0	0.0	Fap	Sex	1.9
11	22	31.8	4.5	63.6	11	9.1	90.9	Fap	Fap	0.4
16	20	45.0	5.0	50.0	–	–	–	Fap	–	0.0
18	25	72.0	0.0	28.0	–	–	–	Sex	–	0.0
20	24	20.8	4.2	75.0	–	–	–	Fap	–	0.0
25	23	95.7	0.0	4.3	20	100.0	0.0	Sex	Sex	8.2
27	29	62.1	3.4	34.5	62	93.5	6.4	Fap	Fap	2.3

behaviour indicated that it is an autotetraploid and this was supported by the chromosome-pairing behaviour in the hybrids recovered when the accession was crossed with the induced autotetraploid *P. plicatulum* line. More importantly, the chromosome pairing in the F_1 hybrids revealed that both species share the same basic genome. However, the higher proportion of univalents observed in tetraploid *P. chaseanum* and the hybrids, than in autotetraploid *P. plicatulum*, suggests that a process of diploidisation might have occurred in the tetraploid cytotypes of *P. chaseanum*.

In spite of the lack of wrinkles on the sterile lemma of the spikelets of *P. chaseanum*, one of the most predominant morphological features of the Plicatula group, our cytological findings support placing this species in the Plicatula group. The affinities of *P. chaseanum* with other *Paspalum* species have also caused some taxonomic controversy. In the original botanical description of the species, Parodi (1932) indicated that the new species was related to *P. virgatum* L. Chase (1939), in her unpublished taxonomic review of the genus for South America, included the species in the Plicatula group, perhaps weighing heavily on the characteristic shining dark-brown anthoecium against the transversely wrinkled glumes. Barreto (1954) placed the species in the Virgata group, a subgeneric informal category of species that share, among other attributes, dark-brown anthoeciums, but lack wrinkles on the glumes. More recently, Zuloaga and Morrone (2005) followed the classification of Chase (1939), a criterion sustained by Giussani *et al*. (2009) based on phylogenetic analyses of DNA data from the chloroplast. Our cytogenetic analyses of *P. chaseanum* and its interspecific hybrids with *P. plicatulum* support the close phylogenetic relation of these species as proposed by Giussani *et al*. (2009), and the inclusion of *P. chaseanum* in the Plicatula group. Similarly, the two species, *P. plicatulum* and *P. chaseanum*, had been included in the same infrageneric classification, named the NPBT-clade, in a phylogenetic analysis of the genus *Paspalum* using sequence data of four non-coding cpDNA fragments (Rua *et al*. 2010). However, this clade included not only a wide array of species of the groups Notata, Plicatula, Bertoniana, and two species of the genus *Thrasyopsis*, but also species with striking differences in morphological and cytological characteristics as for example *P. erianthum* and *P. stellatum*.

The crossability (9.7%) between the two species also indicated that they are closely related. This level of crossability is much higher than that reported among other *Paspalum* species. For example, hybridisation between diploid *P. jurgensii* and a diploid cytotype of *P. intermedium* required the emasculation and subsequent pollination of almost 30 000 florets to recover only three sterile interspecific hybrids (crossability = 0.01%; Burson 1981). Taxonomically, *P. jurgensii* belongs to the Paniculata group and *P. intermedium* to the Quadrifaria group. Moreover, previous indirect crosses have demonstrated that there was no homology between the genomes of these two diploid species because each genome was homologous with one genome of allotetraploids, *P. urvillei* and yellow-anthered *P. dilatatum* (Burson and Bennett 1972; Burson *et al*. 1973; Burson 1979). In comparison with this, crossability was almost 100-fold higher (0.92%) for crosses between diploid *P. notatum* and diploid *P. pumilum* and all the F_1 hybrids recovered were sterile (Quarin and Burson 1983). *P. notatum* and *P. pumilum* were considered to be closely related species and both were in the Notata group (Chase 1929). A cytogenetic analyses indicated that they have partially homologous genomes (Quarin and Burson 1983). Consequently, the crossability of 10% observed in our crosses between *P. plicatulum* and *P. chaseanum*, and the level of fertility observed in some of the hybrids, support a close phylogenetic relationship between these two species.

The fertility and reproductive behaviour of some of the hybrids indicated that there is a possibility that species in the Plicatula group might be improved in a forage-breeding program. Despite the low seed fertility in some of the F_1 hybrids, a few of them were sufficiently fertile to produce additional generations of hybrids and backcrosses, which increases the potential for gene transfer and introgression in a breeding program. Interestingly, the most fertile hybrids were sexual and might be used as maternal parents in a plant-improvement program.

Acknowledgements

We thank Professor Emeritus Henry Fribourg for critically reading the manuscript and for assistance with English. This study was financed by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Grant PIP 11220080101378; Agencia Nacional de Promoción Científica y Técnica, Grant PICT2011 #1802, and Secretaría General de Ciencia y Técnica, Universidad Nacional del Nordeste (UNNE), Grant PI A006, Argentina. Novo was funded by a fellowship from CONICET. Espinoza and Quarin are members of the research staff of CONICET. We thank Florencia Galdeano for her technical assistance concerning flow cytometry and photography.

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