The breeding system of three *Paspalum* species with forage potential

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Abstract

Knowledge of the breeding system is a fundamental step in any program dealing with genetic improvement of wild grasses for forage purposes. This information is essential for *Paspalum*, a large genus with a variety of reproductive methods including apomixis. We conducted cytoembryological studies in 3 species of the group Plicatula (*Paspalum limbatum*, *Paspalum lenticulare* and *Paspalum guenoarum*) in order to understand the breeding systems of 9 accessions which are currently under selection and being bred for forage purposes in our institution. Both accessions of *P. limbatum* were diploid (2n = 2x = 20), showed bivalent chromosome pairing at meiosis and were allogamous due to self-incompatibility. Five accessions of *P. lenticulare* and 2 of *P. guenoarum* were tetraploid (2n = 4x = 40) with mainly bivalent and quadrivalent chromosome pairing at meiosis. The high frequency of quadrivalents suggests autoploidy as the most probable origin for these tetraploids. All these tetraploid accessions showed complete degeneration of the megaspores. The embryo sacs, one to several per ovule, always developed from somatic nucellar cells. These non-reduced embryo sacs produced embryos through parthenogenesis, and were hence genetically identical with the mother plant. Notwithstanding, pollination was required for the development of the endosperm. Only one accession of *P. lenticulare* has some reduced potential for sexual reproduction. Our results indicate that *P. limbatum* is a sexual allogamous diploid species while *P. lenticulare* and *P. guenoarum* reproduce by means of aposporous obligate apomixis.

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Introduction

Paspalum is a large grass genus, including several important forage species which occur in the tropical and subtropical native grasslands in the New World (Chase 1929). Most polyploid species of *Paspalum* so far analysed proved to be apomictic, usually with sexual diploid con-specific counterparts (Quarín 1992). Chase (1929) established several unofficial taxonomic groups for the genus. The group Plicatula contains those species related to *P. plicatulum,* characterised by spikelets with transversely wrinkled lemma and shining dark brown anthecium. Central and western Brazil, eastern Bolivia and Paraguay constitute the geographic centre of variation of this group (Quarín *et al*. 1997). Plicatula may be considered a large agamic complex with considerable forage potential. At least 3 species of this group have been introduced to cultivation: *P. plicatulum*, *P. guenoarum* and *P. atratum*. Oram (1990) listed 3 cultivars of *P. plicatulum* which have been grown in Australia. Ramirez (1954) described "Pasto Rojas", a cultivated form of *P. guenoarum* (sub *P. rojasii*) grown in eastern Paraguay and north-eastern Argentina. *Paspalum atratum,* a tetraploid (2n = 4x = 40) apomictic wild species from Brazil (Quarín *et al*. 1997), has successfully become a cultivated species in tropical and subtropical regions around the world. Cultivars of this species became available during the past decade in USA, Argentina, Thailand and Australia (Kalmbacher *et al*. 1999).

P. lenticulare is the name that Killeen (1990) used for several collections of the Plicatula group from savanna wetlands of eastern Bolivia. It has been considered a synonym of *Paspalum*

plicatulum by most authors since Chase (1929). *P. lenticulare* plants are characterised by folded leaf blades, compressed sheaths and pyramidal or pyramidal-truncate panicles with 5–15 racemes and smaller spikelets than *P. plicatulum*. Plants with these morphological characteristics are common in seasonally inundated savannas in eastern Bolivia, Paraguay and central-western Brazil. Four accessions of *P. lenticulare* from eastern Bolivia proved to be tetraploid (2n = 4x = 40) (Norrmann *et al*. 1994). *Paspalum limbatum* is a small representative of the Plicatula group native from eastern Paraguay, north-eastern Argentina and the Chiquitania region in Bolivia (Quarín 1975; Killeen 1990). Morphologically, *P. limbatum* resembles *P. lenticulare* from which it is distinguished with difficulty, mainly by its smaller inflorescences and spikelets. Three diploid (2n = 2x = 20) accessions of *P. limbatum* were reported from Bolivia (Norrmann *et al*. 1994). Wild *P. guenoarum* grows in Brazil, eastern Bolivia, Paraguay, north-eastern Argentina and Uruguay. In previous studies it was reported to be a tetraploid and an aposporous apomict (Pritchard 1970; Burson and Bennett 1971).

Apomixis, asexual reproduction through seeds (Nogler 1984), gives rise to progenies with a genetic constitution identical with that of the female parent. One of the main advantages of apomixis in plant breeding is that it allows the development of hybrids or genotypes that breed true regardless of heterozygosity. When sexual as well as apomictic individuals of a species are available, new apomictic genotypes could be easily produced through hybridisation because apomictic plants usually produce normal reduced male gametes (Hanna and Bashaw 1987). The knowledge of the method of reproduction is an indispensable step in any program dealing with genetic improvement of wild grasses for forage purposes. There is still a large number of wild *Paspalum* species with outstanding forage potential for which virtually no information on breeding system is available.

The objective of this work was to determine the cytology, the method of reproduction and fertility of 5 accessions of *P. lenticulare,* 2 of *P. limbatum,* and 2 of *P. guenoarum*, in order to assist the characterisation and breeding of these potentially valuable forage grasses.

Materials and methods

The plant material used in these studies belongs to the living *Paspalum* collection held at IBONE, Corrientes, Argentina. The identification numbers and collection sites are listed in Table 1. Those numbers preceded by the letter K were collected and identified by T. J. Killeen who has reported these materials from Bolivia (Killeen 1990). Collection numbers preceded by the letters N and Q have been collected by G. A. Norrmann and C. L. Quarín, respectively.

Young inflorescences of plants growing in a greenhouse or in small field plots were fixed in a 3:1 solution of 100% ethanol:glacial acetic acid and stored in 70% ethanol at 4°C. Pollen mother cells (PMCs) were stained with 2% aceto-carmine and the cells undergoing meiosis were examined using a phase contrast microscope to study meiotic behaviour of each accession. Mode of reproduction was determined by the observation of megasporogenesis and embryo sac development.

Table 1. Origin and identification of the plant material.

Species	Accession no	Collection sites
P. limbatum	N188	Paraguay, 50 km NE of Concepción on route to Paso Barreto
	K2453	Bolivia, Santa Cruz, Prov. Ñuflo de Chávez, Estancia Salta, 10 km S of Concepción on route to Lomerío
P. lenticulare	N153	Paraguay, San Pedro, 10.5 km S of Guayaibí on Route 3
	N164	Paraguay, Amanbay, 33 km S of Bella Vista
	Q4047	Brazil, Goias, 39 km SW of Gaçu
	K2417	Bolivia, Santa Cruz, Concepción
	K2396	Bolivia, Santa Cruz, Prov. Ñuflo de Chávez, Estancia El Recreo, 2 km N of Concepción on route to Lomerío
P. guenoarum	K2390	Bolivia, Santa Cruz, Prov. Ñuflo de Chávez, Estancia El Recreo, 2 km N of Concepción
	K2394	Bolivia, Santa Cruz, Prov. Ñuflo de Chávez, Estancia El Recreo, 2 km N of Concepción

Spikelets were fixed in FAA (90 ml 70% ethanol, 5 ml formaldehyde and 5 ml glacial acetic acid), dehydrated in a butyl alcohol series, embedded in paraffin, sectioned at 12 µm, and stained in a safranin-fast green series. Finally, the slides were observed with a transmission light microscope. In accessions N153 and N164 of *P. lenticulare* only mature embryo sacs were observed with differential interference contrast (DIC) microscopy following the technique described by Herr (1971).

Two techniques, pollen tube growth and percentage of seed set, were used to determine the fertility under self- and open-pollinated conditions for each accession. Pollen tube growth was observed with epifluorescence microscopy following the method of Kho and Baër (1968). Self pollination was performed by confining inflorescences within glassine bags prior to anthesis. In open pollination, inflorescences were bagged after blooming in a place where other genotypes were flowering. Flowering observations for the accessions studied were made in plants growing in a field nursery.

The apomictic accessions were also investigated in order to know whether or not pollination was essential for endosperm development (pseudogamy). Spikelets were emasculated according to the method used by Martínez *et al*. (1994) and inflorescences were bagged after emasculation to prevent undesired pollen reaching the stigmas.

Results

Cytology

Meiotic chromosome configurations at diakinesis and metaphase I of 3 *Paspalum* species involving 9 accessions are shown in Table 2. The 2 accessions of *P. limbatum* were diploid (2n = 2x = 20). The 5 accessions of *P. lenticulare* and both *P. guenoarum* accessions were tetraploid (2n = 4x = 40). Chromosome behaviour at meiosis was regular in both diploid accessions of *P. limbatum*, showing 10 bivalents (Figure 1a), except for one cell that displayed 2 univalents and 9 bivalents in accession K2453. Tetraploid accessions of *P. lenticulare* and *P. guenoarum* showed irregular meiosis, with the chromosomes associating mainly as bivalents and quadrivalents (Figures 1b, 1c and 1d). Univalents and trivalents were also observed in the same pollen mother cells. One hexavalent was observed in K2396. This accession had the highest average of quadrivalent associations per PMC (5.5) with a range of 3–9 per PMC. Lagging chromosomes were often observed at anaphase I in *P. lenticulare* and *P. guenoarum*.

Table 2. Meiotic chromosome configurations at diakinesis and metaphase I analysed in pollen mother cells (PMCs) in different species and accessions of *Paspalum* (I = univalents; II = bivalents; III = trivalents; IV = quadrivalents; and VI = hexavalents).

Species and accessions	2n	PMCs scored (no)	Chromosome associations, mean and range (in parenthesis) per PMC				
			I	II	III	IV	VI
P. limbatum							
N188	20	40	–	10	–	–	–
K2453	20	107	0.02	9.99	–	–	–
			(0–2)	(9–10)	–	–	–
P. lenticulare							
N153	40	52	0.21	12.71	0.02	3.57	–
			(0–2)	(6–19)	(0–1)	(0–7)	–
N164	40	28	0.07	15.25	–	2.35	–
			(0–2)	(10–18)	–	(1–5)	–
Q4047	40	50	0.2	11.8	0.1	4.0	–
			(0–2)	(2–20)	(0–2)	(0–8)	–
K2417	40	59	0.2	11.2	0.1	4.2	–
			(0–2)	(4–18)	(0–2)	(1–8)	–
K2396	40	45	0.4	8.6	0.06	5.5	0.02
			(0–2)	(2–14)	(0–1)	(3–9)	(0–1)
P. guenoarum							
K2390	40	35	1.1	13.7	0.4	2.7	–
			(0–6)	(6–19)	(0–1)	(0–7)	–
K2394	40	67	0.3	11.7	0.04	4.0	–
			(0–4)	(4–18)	(0–1)	(1–8)	–

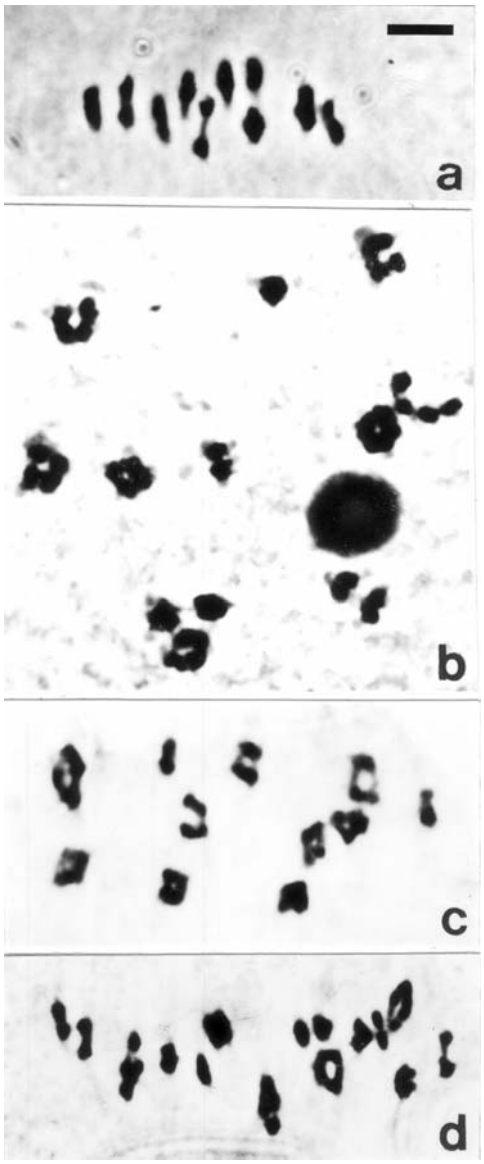

Figure 1. Meiotic chromosome pairing in 3 *Paspalum* species. a) Diploid *P. limbatum*, accession K2453, 10 bivalents; b) Diakinesis in *P. guenoarum,* accession K2394, 6 bivalents and 7 quadrivalents; c) *P. lenticulare*, accession K2696, 2 bivalents and 9 quadrivalents; and d) *P. lenticulare*, accession K2417, 10 bivalents and 5 quadrivalents. Bar = 5 μm.

Mode of reproduction

Megasporogenesis and embryo sac development in diploid accessions of *P. limbatum* were similar to those observed in other diploid *Paspalum* species (Espinoza and Quarín 1997). Meiosis of the megaspore mother cell produced a linear tetrad of megaspores. The three-micropilar members degenerated, and the chalazal one formed the meiotic embryo sac. At maturity, this sac of meiotic origin was characterised by the presence of the egg apparatus (the egg cell and 2 synergids), 1 binucleated central cell and a large number of antipodal cells, which are characteristic of most grasses with sexual reproduction.

All accessions of *P. lenticulare* and *P. guenoarum* showed the typical embryological pathways of other apomictic *Paspalum* species (Quarín and Norrmann 1987). Early development of the megaspore mother cell was usually identical in sexual and apomictic plants. Meiosis in both cases produced a linear tetrad of megaspores. However, in these tetraploid accessions, all megaspores degenerated and the embryo sac developed by mitotic division from nucellar cells (1–4), which were easily distinguished from the surrounding cells because they usually had a dense cytoplasm and a prominent nucleolus. At maturity, aposporous embryo sacs showed 1 egg cell surrounded by 1 or 2 synergids and a binucleated and highly vacuolated central cell. At this stage, the synergid`s nuclei could not be observed and both polar nuclei of the central cell showed a rounded nucleolus with a diameter twice as large as the nucleolus of the egg cell. Antipodal cells were not formed in aposporous sacs and often there was more than 1 embryo sac per ovule, with different shapes, orientation, sizes and developmental stages. The absence of antipodal cells in aposporous embryo sac and mature ovules with multiple embryo sacs per ovule were the most distinguishable embryological characteristic to recognise apomictic plants. Occasionally, a meiotic embryo sac developed in some ovules of *P. lenticulare*, accession K2417, indicating that it has a small potential for sexual reproduction. The other tetraploid accessions of both species are all apparently obligate apomicts.

In addition, pro-embryos were eventually observed at the time of anthesis in aposporous sacs of apomictic species. This early development of the embryo was frequently observed in accession K2417 of *P. lenticulare* even in several embryo sacs of the same ovule (Figures 2a, 2b and 2c). Seventy-five ovules for each of the accessions N153 and N164 were observed in mature ovaries by DIC microscopy and they showed characteristics similar to those described above: absence of

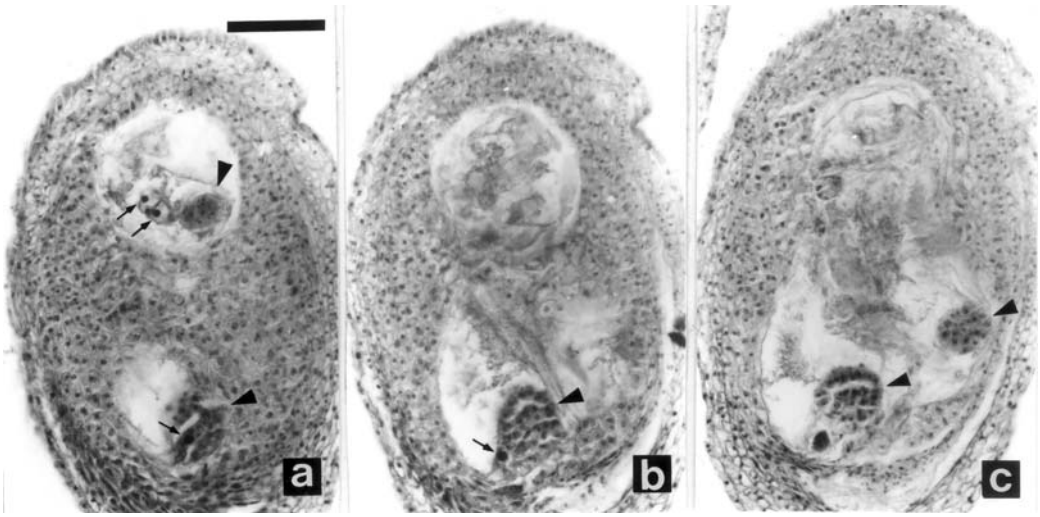

Figure 2. *Paspalum lenticulare*, accession K2417. Three consecutive sections (a, b, and c) of a single mature ovary, fixed at the time of anthesis. The ovule has 3 aposporous embryo sacs: one in the micropilar end (bottom), one in the chalazal region and the third in a lateral position (right). Each embryo sac contains a developing embryo (arrow heads). The presence of the polar nuclei (arrows) indicates that endosperm development would start later, after fertilisation. The polar nuclei of the third embryo sac are in another section (not shown). Bar = 100 µm.

antipodal cells and multiple embryo sacs per ovule.

Flowering and fertility

All the studied accessions had a short flowering period during late summer and early fall. The 2 accessions of *P. limbatum* had different behaviours: accession N188 flowered from the end of February to the end of March whereas in K2453 the flowering occurred from mid-March to mid-April. Both accessions of *P. guenoarum* and all accessions of *P. lenticulare* had coincident flowering periods from the end of April to mid-May. Anthesis in *P. limbatum* and *P. lenticulare* occurred from 13.00 to 15.00 h and in both accessions of *P. guenoarum* from 07.00 to 09.00 h.

Fluorescence studies of pollen-pistil compatibility under self- and cross-pollination conditions are indicated in Table 3. In self-pollinated accessions of *P. limbatum,* the pollen grain germinated, pollen tubes penetrated the stigma papillae but their development was arrested in the upper part of the style. Nevertheless, when different genotypes (individuals) belonging to accession N188 were allowed to cross pollinate, the pollen grains germinated and the pollen tubes penetrated the stigma branches, the style, and then reached the micropyle zone in the ovule. The fact that only one plant of accession K2453 was available barred taking cross-pollination data. In all accessions of *P. lenticulare* and *P. guenoarum*, the pollen grain germination and tube growth were similar in self and cross pollinations. One to 6 pollen tubes were observed reaching the micropilar zone approximately 3 h after pollination following either self or cross pollination.

Seed set was in agreement with the compatibility studies (Table 3). The plant K2453 of *P. limbatum* failed to form seed following self pollination and the seed set was extremely low in accession N188. More than 57% of the spikelets of N188 formed caryopses after cross pollination. On the other hand, tetraploid accessions of *P. lenticulare* and *P. guenoarum* set seed whether selfing or outcrossing had ocurred. The failure of seed formation following emasculation indicated that fertilisation of the central cell was essential to develop the endosperm in aposporous apomictic tetraploid accessions of *P. lenticulare* and *P. guenoarum* (Table 3).

Table 3. Pollen tube growth, percentage of spikelets forming caryopses after self and cross pollination in 3 *Paspalum* species and seed set performance after emasculation in accessions of *P. lenticulare* and *P. guenoarum*.

Species and accesions	2n	Number of pollen tubes penetrating up to the micropyle 3 h after:		Percentage of spikelets that produced seed after:		Seed set after emasculation (bagged inflorescences)	
		Self pollination	Cross pollination	Self pollination	Cross pollination	Emasculated spikelets (no)	Seed set (%)
P. limbatum							
N188	20	0	1–3	0.5	57.2	–	–
K2453[1]	20	0	–	0	–	–	–
P. lenticulare							
N153	40	1–3	2–5	42.9	43.7	54	0
N164	40	1–4	1–4	54.5	40.7	40	0
Q4047	40	1–4	1–3	12.8	21.4	59	0
K2417	40	1–3	1–4	24.6	50.8	52	0
K2396	40	1–4	1–6	11.2	36.6	59	0
P. guenoarum							
K2390	40	1–5	1–4	36.2	33.6	17	0
K2394	40	1–8	1–4	37.8	44.2	158	0

[1] Only one plant of accession K2453 was available so no cross-pollination data could be collected.

Discussion

We are reporting for the first time the meiotic chromosome behaviour and the method of reproduction in 5 tetraploid accessions of *P. lenticulare* and 2 accessions of diploid *P. limbatum*. Apomictic reproduction had been reported for *P. guenoarum* in previous studies (Pritchard 1970; Burson and Bennett 1971) and was confirmed in this work. Our results indicate that *P. limbatum* reproduces sexually and is likely to be allogamous since individual plants of both accessions N188 and K2453 proved to be self-sterile, while plants of K2453 were cross-compatible. The 5 accessions of *P. lenticulare* are apomictic, pseudogamous and self-compatible. The meiotic behaviour and reproductive system of the 2 accessions of *P. guenoarum* studied here agree with those previously reported for this species (Pritchard 1970; Burson and Bennett 1971), since our results indicate that the species is pseudogamous and self-compatible.

The high number of quadrivalent chromosome associations observed in 40-chromosome races of *P. lenticulare* and in *P. guenoarum* suggests that they originated by autotetraploidy. Autoploidy has been suggested as the most likely origin for several apomictic *Paspalum* species (Norrmann *et al*. 1989; Quarín 1992). Autoploidy has been demonstrated by cytological studies in *P. notatum* (Forbes and Burton 1961) and *P. rufum* (Quarín *et al*. 1998), and via genetic analysis in *P. simplex* (Pupilli *et al*. 1997).

The main morphological characteristics used to distinguish *P. limbatum* from *P. lenticulare* refer to the general plant size, and particularly to size of spikelets, which are smaller in *P. limbatum* (Killeen 1990). In addition, *P. lenticulare* has a larger number of racemes per inflorescence. These minor morphological differences and the probable autoploid origin of *P. lenticulare* suggest that *P. limbatum* may be the diploid form of *P. lenticulare*. Further research is needed to determine whether *P. limbatum* and *P. lenticulare* are con–specific. The first step in this research would include artificial diploidisation of *P. limbatum* plants. If induced 4x plants reproduce sexually it should be possible to produce *P. limbatum* × *P. lenticulare* tetraploid hybrids in order to investigate: 1) their cytogenetic relationship; and 2) the feasibility of producing apomictic 4x hybrids with outstanding agronomic characteristics.

Several *Paspalum* species of the Plicatula group are very promising candidates to be used as forage grasses in warm regions of the New World. In fact, some races of *P. guenoarum* (Ramirez 1954 sub *P. rojasii*), *P. plicatulum* (Oram 1990) and *P. atratum* (Kretschmer *et al*. 1994; Quarín *et al*. 1997) have already been brought into cultivation in different countries. The cultivated races of these species have been selected directly from the wild considering their agronomic qualities. Genetic improvement is impracticable because they are apomictic tetraploids. In the genus *Paspalum,* most apomictic species are tetraploid and usually possess sexual

diploid con-specific counterparts (Quarín 1992). Sexual 4x plants have not been found among apomictic wild tetraploid populations (Quarín *et al*. 2001). Thus, doubling the chromosomes of sexual diploids is the most feasible way to produce a 4x sexual plant to be used as the pistilate parent in sexual × apomictic crosses.

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