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ORIGINAL ARTICLE

Ploidy levels and reproductive behaviour in natural populations of five *Paspalum* species

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Abstract Knowledge of variation in ploidy levels and reproductive behaviour in natural populations is essential in order to understand the functioning of agamic complexes. The aim of this study was to analyse the ploidy level and mode of reproduction in several wild *Paspalum* populations. A total of 19 populations representing five different species (*P. alcalinum*, *P. denticulatum*, *P. lividum*, *P. nicorae*, and *P. rufum*) were collected. Ploidy level was determined in 1,187 individuals by using flow cytometry. Among these individuals, 2x, 3x, 4x, 5x, 6x, and 7x chromosome constitutions were observed. Diploid sexual cytotypes of *P. denticulatum* were detected for the first time; this will allow the development of future breeding strategies for this particular species. Flow cytometry seed screen (FCSS) in bulked and single seeds revealed the reproductive diversity of these species, ranging from complete sexuality in diploids and varying levels of facultative apomixis in most tetraploids, to obligate apomixis in pentaploids and hexaploids. A fully sexual tetraploid plant was never detected. Nevertheless, most tetraploid genotypes produced both maternal (by apomixis) and non-maternal (by sexuality) progeny. This residual sexuality is very interesting from an evolutionary point of view, since it would allow the creation of new genotypic combinations in natural populations. In addition, the residual sexuality found in some apomictic tetraploid populations can be used as a source of variability for genetic improvement.

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Introduction

Species of the grass genus *Paspalum* are major constituents of native pasturelands in tropical and subtropical regions of the Americas. In this genus, the considerable morphological diversity and the wide range of adaptability to different environments are unquestionably sustained by its reproductive diversity, which has a large evolutionary influence in this plant group (Bashaw et al. 1970). *Paspalum* includes sexual diploids but comprises mainly sexual and apomictic polyploid species (Quarin 1992). Polyploidy is a common feature present in about 80% of species, varying from 3x (Quarin and Lombardo 1986) to 16x (Burton 1940). Among all ploidy levels, tetraploidy is the most frequent condition. Apomixis, asexual reproduction through seeds (Nogler 1984), and polyploidy, constitute the most common combination for most *Paspalum* species. In apomictic individuals, fertilisation of the non-reduced egg cell is bypassed, and maternal clonal embryos are generated by parthenogenesis, while the two non-reduced polar nuclei of the central cell are fertilised by one sperm cell to develop the endosperm (pseudogamy).

It is generally observed that hybridisation and polyploidy represent two important processes in the evolution of apomictic angiosperms. However, the relative contribution of these processes is not clear. Ernst (1918) sustained that all apomicts are of hybrid origin, and Stebbins (1941) added that the great majority of apomicts are probably allopolyploids, and therefore ultimately of hybrid origin. Similarly, Nogler (1984) considered that alloploidy is just as typical for apomicts as hybridity. More recently,

these concepts have been referred as to the 'hybridisation theory' (Carman 1997) to explain the appearance of apomixis. This theory indicates that hybridisation of two species with different reproductive characters may contribute to the induction of apomixis. The hybrids contain two sets of parental genes that are involved in embryo sac development and embryogenesis. The asynchronous expression of these duplicated genes leads to initiation of embryological processes at aberrant sites and times during reproduction, thus producing the necessary embryological changes to drift the process from sexual to apomictic reproduction.

In the genus *Paspalum*, polyploidy seems to be a prerequisite for the effective expression of apomixis (Quarin and Hanna 1980; Quarin et al. 2001), but apomixis is not necessarily accompanied by hybridity. Support for these assertions comes from cytogenetic studies of several polyploid species, from segregation analyses carried out in apomictic tetraploid species, and from synthetic induction of apomictic tetraploids from sexual diploids. Cytogenetic studies have revealed that a high proportion of chromosomes form multivalent chromosome associations in apomictic polyploids, suggesting an autoploid origin (see for example Norrmann et al. 1989; Hojsgaard et al. 2008). Genetic analysis has revealed tetrasomic inheritance in apomictic tetraploids, as is typical of autoploids (Pupilli et al. 1997; Stein et al. 2004). In addition, synthetic polyploidisation induced from sexual diploids triggered and maintained apomictic reproduction in autotetraploid *Paspalum* species (Quarin and Hanna 1980; Quarin et al. 2001). These latter studies are just examples, among many others, supporting the hypothesis that most apomictic *Paspalum* species originated by autoploidy from sexual diploid relatives.

Recurrent auto-polyploidisation through $2n + n$ fertilisation events has been documented by experimental work in *Paspalum rufum* involving sexual diploids, which have some capacity for apospory, apomictic triploid bridges, and apomictic autotetraploids (Siena et al. 2008). Thus, *Paspalum* constitutes a very important plant group among angiosperms in relation to plant evolution and apomixis. It has been estimated that over 400 species from 40 different families of flowering plants (approximately 0.1% of angiosperms), have evolved an asexual form of seed formation termed apomixis (Tucker and Koltunow 2009). *Paspalum* comprises approximately 330 species (Zuloaga and Morrone 2005). The method of reproduction has been investigated only in 20% of these species, with 40 species being found to be apomictic. This means that approximately 10% of apomictic angiosperms belong to the genus *Paspalum*. There are some *Paspalum* apomicts for which a hybrid origin has been well documented, as in the case of tetraploid (postrate biotype), pentaploid and hexaploid cytotypes of *P. dilatatum* (Bennett and Bashaw 1966; Burson 1983, 1991) among few other species. However, most apomictic *Paspalum* species belong to a model system characterised by co-specific diploid-autopolyploid taxa. Diploids are sexual outbreeders with some potential for occasional aposporous embryo sac formation (Norrmann et al. 1989), and autopolyploids (mainly tetraploids) are aposporous pseudogamous apomicts. The hypothesis is that, in this model system, the apomictic species constitute agamic complexes with sexual diploids, occasional apomictic triploids, frequent apomictic tetraploids, and additional apomictic pentaploids, hexaploids and heptaploids. To test this assumption, we examined natural populations of five *Paspalum* species from different geographic origins, analysing ploidy levels and reproductive mode.

Chase (1929) included *P. alcalinum* Mez, *P. denticulatum* Trin., and *P. lividum* Trin. into the Livida group—an informal subgeneric taxonomic rank. Although *P. lividum* has been referred to as a synonym of *P. denticulatum* (Denham et al. 2010), we prefer to follow the original Chase criterion since convincing evidence for synonymous treatment has not been provided. *Paspalum alcalinum* is a valuable indigenous forage grass with sexual diploid ($2n = 2x = 20$), facultative apomictic tetraploid ($2n = 4x = 40$), obligate apomictic pentaploid ($2n = 5x = 50$) and hexaploid ($2n = 6x = 60$) cytotypes (Burson 1997; Hojsgaard et al. 2009). *Paspalum denticulatum* is native to western Brazil, Paraguay and northern Argentina. Since facultative apomixis and autotetraploidy have been reported for this species (Quarin and Burson 1991), other ploidy levels and reproductive modes might be expected. *Paspalum lividum* is a stoloniferous perennial grass for which tetraploidy and apomixis have been reported repeatedly (Burson and Bennett 1971; Quarin 1977; Pagliarini et al. 2001; Gould 1958, 1968; Reeder 1967; Davidse and Pohl 1972). In addition, a heptaploid ($2n = 7x = 70$) accession has been registered earlier (Snyder 1953). *Paspalum nicorae* Parodi belongs to the Plicatula group, and was described as tetraploid ($2n = 4x = 40$) with a pseudogamous aposporous apomictic mode of reproduction (Burson and Bennett 1970). Information for a large number of tetraploid accessions has been published (Moraes Fernandes et al. 1974; Pagliarini et al. 2001), including a list of more than 50 accessions collected throughout the state of Rio Grande do Sul, Brazil (Reis et al. 2010). *Paspalum rufum* Nees involves diploid races ($2n = 2x = 20$) that reproduce sexually (Saura 1948; Norrmann et al. 1989) and tetraploid races ($2n = 4x = 40$) that reproduce by means of facultative apomixis (Burson 1975; Moraes Fernandes et al. 1974; Norrmann et al. 1989).

Although different ploidy levels and reproductive modes have been described for the five species of *Paspalum* mentioned above, the corresponding information has been

gathered usually from one or only a few plants of each accession. The objective of this work was to analyse the ploidy level among individuals of natural populations, and the mode of reproduction across ploidy levels and among individuals sharing the same chromosome number.

Materials and methods

Plant material

Nineteen populations involving five species of *Paspalum* were collected from a wide range of their natural geographic distribution in Argentina, including two localities of Paraguay for *P. denticulatum* and *P. alcalinum* (Table 1, Fig. 1). Each population was sampled by collecting rhizome pieces from single plants in their natural environment. These samples were taken at least 10 m apart one from each other to avoid sampling the same individual twice. A minimum of 10 and a maximum of 90 individual cuttings were collected per population, though collections ranged from 50 to 90 individuals for most populations. The cuttings were grown in pots in a greenhouse for ploidy level determination. In some populations, mature seeds were also harvested in the field from ten randomly selected plants in order to analyse their mode of reproduction. When seeds were not available at the time of field collection, the plants were allowed to flower in the greenhouse and seed samples were harvested from ten individuals per population. Herbarium specimens for each population were collected in the field and deposited at CTES herbarium (Instituto de Botánica del Nordeste, CONICET, Corrientes, Argentina).

Ploidy determination

Flow cytometry was used to determine the ploidy level of each individual from the 19 populations. The fluorescence intensity of DAPI-stained nuclei was analysed with a Partec PA II (Partec, Münster, Germany) flow cytometer. The ploidy level of each individual was determined using samples of fresh leaf tissue following the recommendations of the Partec P kit CySatin UV Precise P 05-5002 manual. Briefly, 0.5 cm^2 leaf material was placed in a small Petri dish with a similar amount of tissue from the control (a plant of the same species for which the chromosome number was established by chromosome counts in root tips). After adding extraction buffer (0.5 ml), the tissue was chopped with a sharp razor blade. Following a 2-min incubation, samples were filtered through a 50 μm nylon mesh directly into the sample tube, to which 1.5 ml DAPI (4′,6-diamidino-2-phenylindole) stain solution (Partec P kit

Table 1 Identification, collection sites and ploidy levels of individuals originating from different populations of *Paspalum* species, as revealed by flow cytometry. *A* Argentina, *P* Paraguay

Species	Herbarium voucher	Population	Locality of collection	Number of plants analysed	Number of individuals with					
					2x	3x	4x	5x	6x	7x
P. alcalinum	MS1	A1	A. Chaco, Tres Isletas	57				48	8	1[a]
	MS10	A2	A. Salta, El Galpón	10					10	
	MS13	A3	A. Chaco, Antequeras	64					64	
	Q4303	A4	P. 120 km NW of Asunción	35					35	
P. denticulatum	MS2	D1	A. Chaco, Tres Isletas	60			60			
	MS12	D2	A. Chaco, Antequeras	73			73			
	Q4304	D3	P. 52 km NW of Asunción	52	42[a]	2[a]	8			
P. lividum	MS8	L1	A. Salta, Orán	63			63			
	MS9	L2	A. Salta, El Galpón	72			72			
	MS26	L3	A. Corrientes, Riachuelo	44			44			
P. nicorae	MS4	N1	A. Corrientes, Santa Ana	88			88			
	MS5	N2	A. Santa Fe, Cayastá	84			84			
	MS7	N3	A. Corrientes, Mantilla	90			90			
P. rufum	MS6	R1	A. Corrientes, Paso Rosario	74	1		73			
	MS16	R2	A. Chaco, 50 km N of Resistencia	83	83					
	MS18	R3	A. Santa Fe, Gobernador Crespo	70	15		55			
	MS20	R4	A. Entre Ríos, Concordia	62			62			
	MS22	R5	A. Corrientes, Paso Lucero	45	45					
	MS23	R6	A. Corrientes, Saladas	61	61					

[a] New record for chromosome number

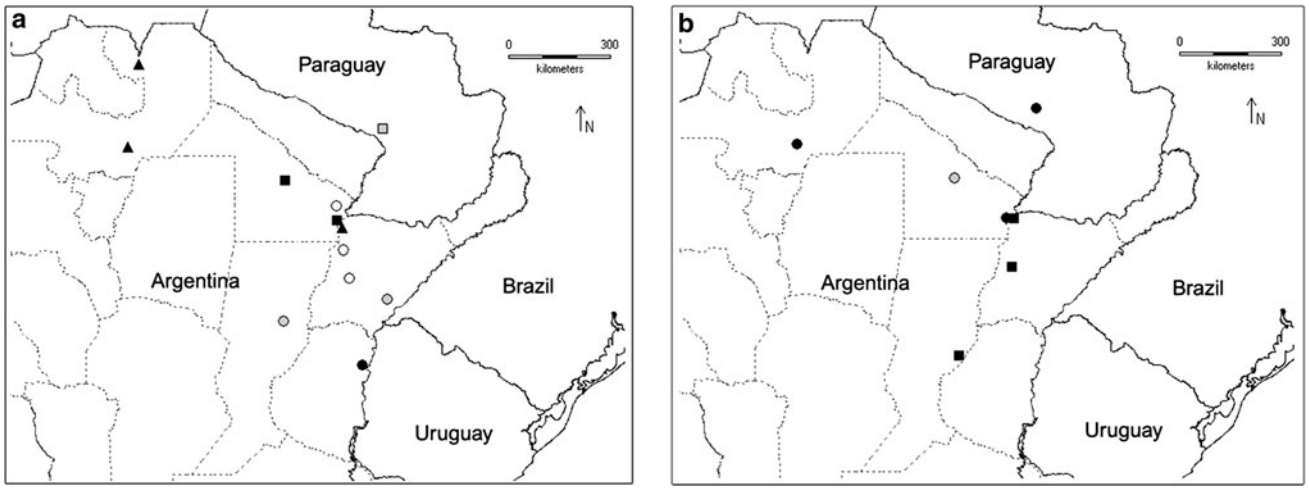

Fig. 1 Geographical distribution of natural populations of five species of *Paspalum* used in this study. **a** *P. rufum* (2x = *white circles*; 2x/4x = *grey circles*; 4x = *black circle*); *P. denticulatum* (2x/3x/4x = *grey square*; 4x = *black squares*) and *P. lividum* (4x = *black triangles*); **b** *P. alcalinum* (5x/6x = *grey circle*; 6x = *black circles*) and *P. nicorae* (4x = *black squares*)

CySatin UV) was added. The mixture was incubated for another 2 min at room temperature and analysed. Ploidy levels were estimated in relation to the DNA peaks in the samples and the internal standard. The plants were measured once, but in case of doubt measurements were repeated two or more times. In addition, results were confirmed by counting somatic chromosome numbers in root tips in representative plants of each population. Root tips were removed from potted plants, fixed in a saturated solution of α-bromonaphthalene for 2 h and hydrolyzed in 1.0 mol m^{-3} HCl for 10 min at 60°C. Root tips were stained with 20 mol m^{-3} basic fuchsin, squashed with a drop of aceto-orcein on slides and observed with a phase contrast microscope.

Mode of reproduction

The reproductive behaviour within each population was determined by flow cytometry according to Matzk et al. (2000). The flow cytometric seed screen (FCSS) method allows sexual seed to be distinguished from apomictic seed origin by comparing the embryo and endosperm DNA content. Assuming that the C-value refers to the entire nuclear DNA in the haploid chromosome complement (n) of a plant, and taking advantage of flow cytometry facilities to quantify the DNA embryo and endosperm tissues in seeds, it is possible to establish the reproductive mode that gave rise to a particular seed of *Paspalum* species. Seeds which originated sexually show a 2C:3C embryo/endosperm ratio, whereas a 2C:5C ratio corresponds to those produced by pseudogamous apomixis. Histograms from flow cytometry regularly show a 4C peak in addition to the 2C and 3C or 2C and 5C peaks. The 4C peak represents embryo cells at the G2 stage of the cell cycle. Initially, 6–10 individuals per population were selected randomly to determine the mode of reproduction. A bulk of 10 caryopses of each selected plant was then analysed. A flow cytometric histogram with two main peaks equivalent to 2C and 3C values indicated that the genotype reproduced sexually (embryo = $n + n$; endosperm = $n + n + n$). A histogram showing two main peaks corresponding to 2C and 5C values revealed an apomictic origin (embryo = $2n + 0$; endosperm = $2n + 2n + n$, i.e., apospory + parthenogenesis + pseudogamy). If the 10-seed bulk analysis of some plants generated a histogram with three main peaks, i.e., 2C, 3C, and 5C, an extra 20-seed sample was analysed, seed by seed, in two of these plants per population.

Ten caryopses were separated from spikelets for bulk seed analysis (BSA), finely chopped in nuclear extraction buffer and filtered through a 30 µm mesh following the recommendations of the Partec P kit CySatin UV as described for leaf tissue. When needed, a supplementary single seed analysis (SSA) was conducted in order to split up, in a sample of two individuals, the 2C, 3C, and 5C peaks of histograms obtained from bulked samples.

Results

Population ploidy level

The ploidy level of 1,187 plants from 19 populations was determined by flow cytometric analysis (Table 1). Four populations of *P. alcalinum* were analysed. In three of them, only hexaploid ($2n = 6x = 60$) plants were found, while in population A1, individuals with different ploidy levels were detected. Most of the 57 plants analysed were

pentaploids (84.2%), 14% were hexaploids, and 1 plant (1.8%) was heptaploid. Three populations of *P. denticulatum* were examined, and all individuals from populations D1 and D2 were tetraploids. However, population D3 (52 individuals) showed diploid (80.77%), triploid (3.85%) and tetraploid (15.38%) cytotypes (Fig. 2a–c). This is the first report of diploid and triploid cytotypes for this species. Individuals from *P. lividum* and *P. nicorae* populations showed similar ploidy levels: 441 plants were analysed and all turned out to be tetraploids. Six *P. rufum* populations were investigated: only diploid plants were recovered from populations R2, R5, and R6, and exclusively tetraploids were observed in population R4, while populations R1 and R3 showed diploid and tetraploid individuals co-existing in the same area. In both mixed populations, tetraploid plants predominate and the frequency of diploids plants was 1.3 and 21.4% for R1 and R3, respectively. Triploid plants were not recovered in native populations of *P. rufum*.

Mode of reproduction

The relative DNA content observed by FCSS in bulks of ten seeds for ten randomly selected plants per population substantiated the classification of reproductive mode summarised in Table 2. Results indicated that pentaploid and hexaploid *P. alcalinum* cytotypes reproduce by apomixis. All histograms generated by BSA displayed at least two evident peaks corresponding to 2C embryo and 5C endosperm values (Fig. 3a,b). In apomictic species of *Paspalum*, the aposporous embryo sac usually contains four cells and five unreduced ($2n$) nuclei: the egg cell, two synergids, and a large bi-nucleated central cell bearing two polar nuclei. The embryo (2C value) develops from the egg cell by parthenogenesis ($2n + 0$), and the endosperm (5C value) arises through pseudogamy as a result of fertilisation of the two polar nuclei by a single reduced sperm nucleus ($2n + 2n + n$). In addition, several histograms from plants of A2 and A3 populations displayed a third 3C peak. Since the histograms were built by BSA from a 10-seed sample per plant, a total of 40 seeds from two of these plants per population were then analysed by SSA. The supplementary 40 single-seed histograms fell into two categories: those having 2C and 5C peaks, and those which showed 3C and 5C peaks. The interpretation was that histograms with 2C and 5C peaks were typical of seed that had originated by means of apomixis: embryo $2n + 0$ (apospory + parthenogenesis) and endosperm $2n + 2n + n$ (apospory + pseudogamy). On the other hand, histograms with 3C and 5C peaks were consistent with the formation of B_{III} seed through fertilisation of an unreduced female gamete by a reduced sperm nucleus ($2n + n$), due to failure of parthenogenesis in an aposporous sac (Fig. 3c). This means that double fertilisation occurred in some aposporous embryo sacs (apospory + double fertilisation; embryo $2n + n$ and endosperm $2n + 2n + n$).

Bulk seed analysis of two pure 4x populations of *P. denticulatum* (D1 and D2) showed histograms with main peaks at 2C, 3C, and 5C values, similar to the histograms observed for some plants of *P. alcalinum*. Then, SSA conducted on two plants per population (20 seeds of each plant) resulted in three different histogram types: most individual seeds showed 2C and 5C peaks indicating their apomictic origin; some single seeds produced histograms with 2C and 3C peaks because they had a sexual origin, i.e., double fertilisation in a sexual embryo sac. Hence the embryo had a 2C value ($n + n$) and the endosperm 3C ($n + n + n$). The third type of histogram had two main

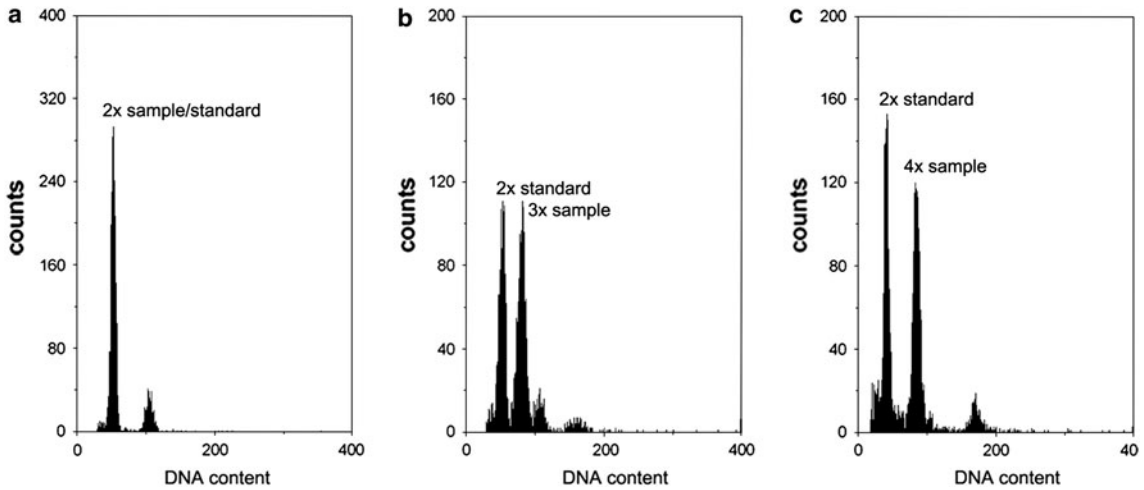

Fig. 2 Histograms from cell nuclei of leaves samples showing different ploidy levels in three cytotypes of *Paspalum denticulatum*. **a** Diploid ($2n = 2x$); **b** triploid ($2n = 3x$); and **c** tetraploid ($2n = 4x$)

Table 2 Reproductive mode of five *Paspalum* species inferred from embryo/endosperm DNA content ratio by flow cytometric seed analyses: $n + n$ = sexuality; $2n + 0$ = apospory + parthenogenesis + pseudogamy; $2n + n$ = apospory + fertilisation (B_{III})

Species and populations	Ploidy level	Analysed plants (no.)	Plants that originated seed by			Residual sexuality (%)[a]	Proportion of B_{III} embryos in the seed sample (%)	Reproductive mode[b]
			$n + n$	$2n + 0$	$2n + n$			
P. alcalinum								
A1	5x	6		+				A
	6x	4		+				A
A2	6x	10		+	+		7.5 (9x)	A
A3	6x	10		+	+		5.0 (9x)	A
A4	6x	10		+				A
P. denticulatum								
D1	4x	10	+	+	+	17.5	17.5 (6x)	Af
D2	4x	10	+	+	+	23.6	12.5 (6x)	Af
D3	2x	6	+					S
	3x	1		+				A
	4x	2	+	+	+	8.3	12.5 (6x)	Af
P. lividum								
L1	4x	10	+	+		30.0		Af
L2	4x	10	+	+		na		Af
L3	4x	–				na		na
P. nicorae								
N1	4x	10	+	+	+	5.0	32.5 (6x)	Af
N2	4x	10	+	+	+	5.0	15.0 (6x)	Af
N3	4x	10	+	+	+	2.5	15.0 (6x)	Af
P. rufum								
R1	2x	–				na		na
	4x	10		+	+		6.25 (6x)	A
R2	2x	10	+					S
R3	2x	3	+					S
	4x	7		+				A
R4	4x	10		+	+		5.0 (6x)	A
R5	2x	10	+					S

[a] Proportion of seeds formed through sexual means ($n + n$), average of two randomly sampled plants per population

[b] Mode of reproduction (*A* apomictic, *Af* facultative apomictic, *S* sexual, *na* not analysed)

peaks at 3C and 5C values indicating that those seeds had a B_{III} origin as described above for *P. alcalinum*. Thus, these two pure tetraploid *P. denticulatum* populations are facultative apomictic with residual sexuality, and with the potential to increase in ploidy to the hexaploid level. Analysis of single seeds revealed that 23.6 and 17.5% of seed was produced by sexual means in plants of the D1 and D2 populations, respectively. Both populations also formed B_{III} seeds (Table 2). Nine individuals of the mixed population D3, from Paraguay were analysed by BSA: six plants of the diploid cytotype reproduce exclusively sexually, showing histograms with 2C and 3C peaks. One triploid plant produced seeds that resulted in histograms with the 2C and 5C peaks typical of aposporous apomictic reproduction (Fig. 3d). Seed of two tetraploid plants of the D3 population exhibited histograms with three main peaks, 2C, 3C, and 5C, when examined with BSA. Individual analysis of seeds of these tetraploids showed 8.3% of residual sexuality (2C and 3C peaks) and 12.5% of hexaploid B_{III} seeds.

The reproductive behaviour was determined for only two 4x populations of *P. lividum*: L1 and L2. Bulked seeds analysis always showed histograms with 2C, 3C, and 5C values in both populations. SSA was conducted for two plants of the L1 population, and revealed that 30% of seed was formed by sexual means and the remaining 70% by apomixis. No B_{III} seeds were recovered in this population. We were unsuccessful with SSA in L2 and BSA in L3 populations due to insufficient seed stock. The results indicated that tetraploid *P. lividum* reproduced by

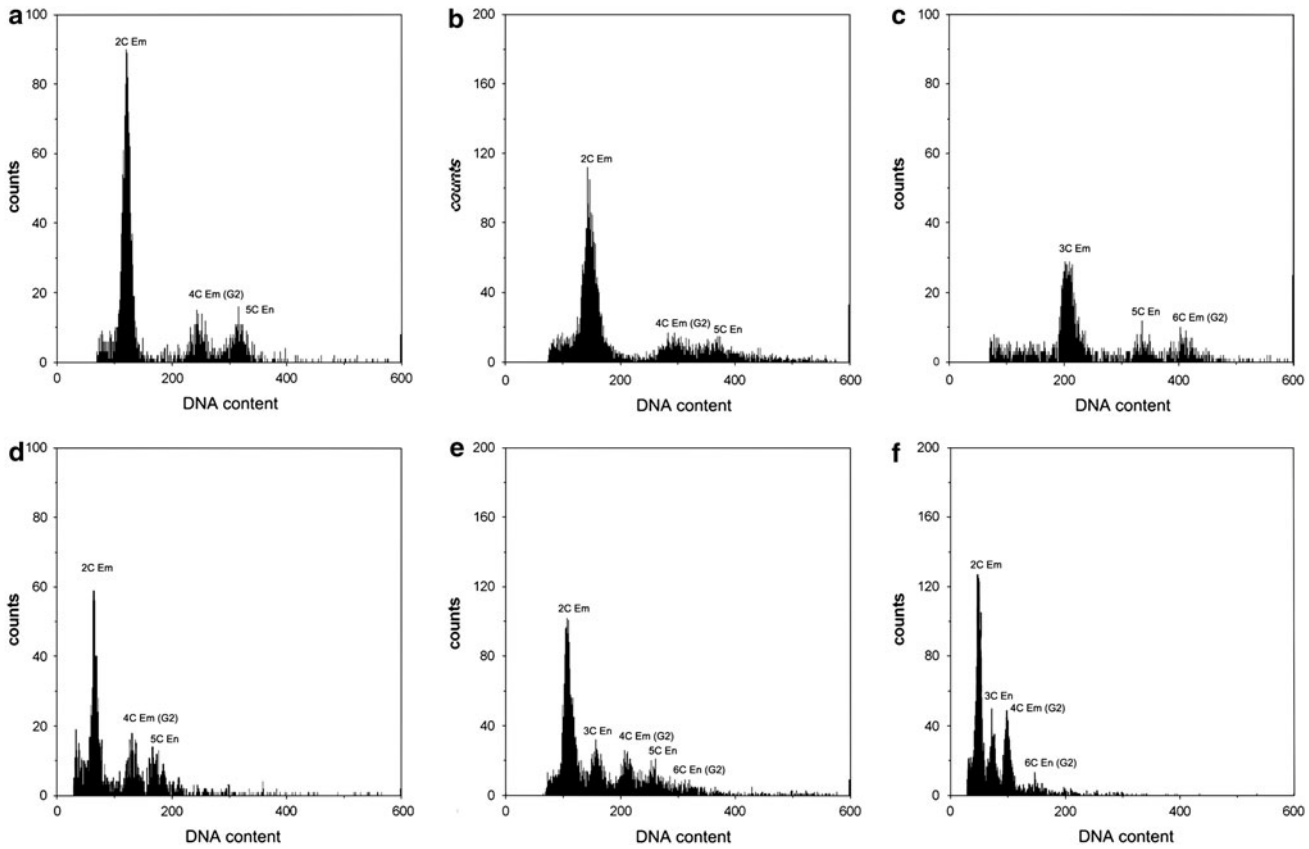

Fig. 3 Histograms from cell nuclei of bulked and single seed samples of *Paspalum* species. Bulked samples (ten seeds) from a pentaploid apomictic 2C/5C (**a**), and a hexaploid apomictic 2C/5C of *P. alcalinum* (**b**). Single seed from a hexaploid plant 3C/5C (B_{III} hybrids) of *P. alcalinum* (**c**) and an apomictic triploid (2C/5C) of *P. denticulatum* (**d**). Bulked samples (ten seeds) from a tetraploid facultative apomictic plant (2C/3C and 2C/5C) of *P. nicorae* (**e**), and a diploid sexual (2C/3C) of *Paspalum rufum* (**f**). *Em* embryo, *En* endosperm

apomixis, probably of the facultative type, as judged according our findings in population L1.

Three populations of *P. nicorae* were examined by BSA (Fig. 3e). The SSA results indicated that the species reproduces by facultative apomixis with 5% residual sexuality in population N1 and N2 and 2.5% in N3. An unexpected high proportion of seeds was formed after $2n + n$ fertilisation (B_{III} seeds), ranging from 15% in populations N2 and N3 to 32.5% in population N1.

Diploid individuals from three pure 2x and one mixed 2x/4x populations of *P. rufum* always showed histograms with 2C and 3C peaks and were therefore classified as sexual (Fig. 3f; Table 2). On the other hand, BSA of tetraploid genotypes from 2x/4x mixed population R3 generated histograms with only 2C and 5C peaks, while tetraploids from populations R1 and R4 showed 2C, 3C, and 5C values. SSA resulted in values indicating that some 4x individuals of populations R1 and R4 occasionally produce B_{III} seed (Table 2). The method of reproduction could not be analysed for the single 2x plant found in population R1 because this plant died before flowering. The results indicated that the 2x cytotype of *P. rufum* reproduces sexually and 4x cytotype reproduces by means of apomixis. No residual sexuality ($n + n$) was observed among tetraploids, though B_{III} seeds were occasionally formed in two populations.

In addition, ploidy level was investigated in plantlets derived from seeds of tetraploid populations N1 of *P. nicorae* and D1 of *P. denticulatum*. Flow cytometry in young leaves revealed that 30.5 and 11% of the progeny of *P. nicorae* and *P. denticulatum*, respectively, were hexaploids, revealing a B_{III} origin. These values are in accordance with the proportion of B_{III} seed formed in N1 (32.5%) and D1 (17.5%), according to our SSA with flow cytometry (Table 2).

Discussion

Most *Paspalum* species involve sexual diploid as well as apomictic polyploid cytotypes. Apomixis and polyploidy, mainly tetraploid, constitute the most common condition in

this genus (Quarin 1992). Although *Paspalum* has a wide range of genetic systems, studies so far have most often been performed on a single or very few plants. Actually, flow cytometry provides an excellent alternative to analyse ploidy level and the reproductive behaviour of individuals within natural populations, representing a cost-effective method to examine numerous plants in a very short time.

Our results revealed the large variability in ploidy levels and reproductive behaviour among individuals of 19 populations comprising five different *Paspalum* species. Diploid populations reproduce sexually, while polyploids, 3x, 4x, 5x and 6x, reproduce by apomixis. Although apomixis was the reproductive system for all tetraploid populations analysed, we observed that most tetraploid plants presented some degree of residual sexuality, which varied from 2.5% in *P. nicorae* (N3 population) to 30% in *P. lividum* (L1 population). In a different way, residual sexuality was not observed in triploids, pentaploids and hexaploids cytotypes. However, B_{III} seeds ($2n + n$) were formed in at least ten populations. This result indicates that elements of apomictic development, such as apospory and parthenogenesis, may be uncoupled, at least occasionally. If this means that apospory and parthenogenesis are controlled by separate loci is still a matter for further research in *Paspalum*.

An example of two independent loci controlling diplospory and parthenogenesis has been proposed in *Erigeron*, a genus of the Asteraceae family (Noyes and Riesenberg 2000; Noyes 2006). *Erigeron* represents a different model system for apomictic pathways. The most important difference in apomictic development in *Erigeron* and in the *Paspalum* species studied here is the first element of the whole process. In apomictic *Erigeron* there is a clear dichotomy in megagametophyte development (meiotic vs diplosporous) and these processes are mutually exclusive. Apomixis is of the aposporous type for most species of *Paspalum*. Meiotic and aposporous megagametophyte development may coexist in the same ovule, and also fertilisation and parthenogenesis might occur in the same ovule. In *Paspalum*, pollination is always necessary for further development, either in meiotic or in aposporous megagametophyte because pseudogamy (fertilisation of the 2-nucleated central cell) is required for seed development. Because of these differences between diplosporous and aposporous systems, it would be interesting to know whether the occurrence of B_{III} hybrids in *Paspalum* means that apospory and parthenogenesis are controlled by different loci. In addition, our results showed that fully sexual plants were not detected at the tetraploid or other polyploid levels, in accordance with previous reports performed in natural populations of *P. simplex* (Urbani et al. 2002) and *P. notatum* (Daurelio et al. 2004).

Although 4x sexual genotypes have not been identified in nature, they have been recovered after colchicine doubling of chromosome numbers in three *Paspalum* species: *P. notatum* (Burton and Forbes 1960; Quarin et al. 2001), *P. simplex* (Cáceres et al. 1999), and *P. plicatulum* (Sartor et al. 2009). Similarly, two facultative apomictic plants were recovered for *P. notatum* after chromosome doubling of different diploid genotypes (Quarin et al. 2001). Additionally, previous reports based on genetic and molecular studies concluded that apospory has a monogenic inheritance with a distorted segregation ratio, and that the locus is situated in a genomic region characterised by a severe restriction of recombination (Martínez et al. 2001, 2003; Stein et al. 2007). Apomixis is controlled by a dominant factor, as was postulated for *P. notatum* (Martínez et al. 2001), for other grass species like *Panicum maximum* (Savidan 1975) and *Brachiaria* sp. (Ndikumana 1985; Valle et al. 1994), or for species of other plant families like *Ranunculus auricomus* (Nogler 1975). On the other hand, our results support the existence of residual sexuality at least in tetraploid apomicts, in agreement with the suggestion of Asker and Jerling (1992), which questions whether any 100% obligate apomicts exist. If apomixis is controlled by a dominant allele and residual sexuality exists in an apomictic population, further research is required to determine why 100% sexual individuals are absent in wild populations of apomictic *Paspalum* species.

A polyploidisation model was described that is applicable to several species of *Paspalum* (Quarin 1992). This model comprises co-specific sexual diploids, rare apomictic triploids and apomictic tetraploids, which usually constitute the most common cytotype for the species. Sexual diploids develop reduced (n) embryo sacs as a consequence of megasporogenesis. Nevertheless, in several species of *Paspalum*, some diploid individuals eventually produce aposporous embryo sacs in the same ovule besides the normal meiotic sac (Norrmann et al. 1989). Studies performed by Siena et al. (2008) showed that these unreduced embryo sacs may be functional and, when fertilised by a reduced male gamete, they give rise to triploid plants ($2x + x = 3x$). These triploids may, in turn, be the origin of new tetraploids ($3x + x = 4x$). Otherwise, the $2n$ gamete of an additional aposporous sac can be fertilised by a male haploid gamete from a neighbouring apomictic 4x plant ($2n$) to establish a new apomictic 4x genotype, as was achieved experimentally in *P. rufum* (Norrmann et al. 1994).

Strikingly, the experimental work of Siena et al. (2008) showed that a diploid plant, when exposed simultaneously to its own reduced haploid pollen ($n = x$) and reduced diploid pollen ($n = 2x$) from a co-specific tetraploid, produced polyploid descendants in only two different ways: 82% were triploids from self-fertilisation of unreduced

egg cells (2x) by a reduced male (x) gamete, and 18% were tetraploid that resulted from cross-fertilisation of unreduced egg cells (2x) by a reduced male (2x) gamete. None of the polyploid descendants were formed by cross-pollination of a reduced female (x) by a reduced male (2x) gamete. These previous experimental results suggest that, in 2x–4x mixed populations, recurrent polyploidisation may take place through fertilisation of unreduced gametes of diploid plants rather than through fertilisation of reduced female gametes of diploid plants by pollen of tetraploid representatives. Depending upon the ploidy level of the pollen source ($n = x$ from diploids or $n = 2x$ from tetraploids) the resulting polyploid would be a triploid or a new tetraploid. Remarkably, the triploids we found in the mixed 2x–3x–4x-population of *P. denticulatum* reproduced by apomixis, as in other triploid cytotypes of different *Paspalum* species (Quarin et al. 1989). Since the experimental work suggests that triploids are formed exclusively by gametes of diploids (unreduced egg cell fertilised by reduced sperm nucleus), it can be assumed that those diploid plants that eventually form an aposporous sac besides the normal sexual one most likely contain the gene/s for apomixis, though they barely express the trait at the diploid level. However, polyploidisation might lead to the normal expression of apomixis, as occurred when new tetraploids were colchicine-induced from some sexual diploid plants of *P. notatum* (Quarin et al. 2001). In fact, the occurrence of triploid bridges was proposed to be one of the most prominent mechanisms of polyploid formation in natural populations (Ramsey and Schemske 1998). Similarly, neotetraploids may also be generated by the union of diploid male gametes from tetraploids and unreduced female gametes from diploids, as described for other species (Woodell and Valentine 1961; Bretagnolle and Lumaret 1995; Siena et al. 2008). Our results regarding *P. denticulatum* and *P. rufum* are in agreement with the hypothesis of recurrent autopolyploidisation, based upon fertilisation of unreduced gametes involving occasional aposporous sacs formed in diploid cytotypes. Thus, the combination of apomixis and tetraploidy constitutes, for these two species, and likely for many other *Paspalum* species, the most effective condition to be successful.

The populations of *P. nicorae* and *P. lividum* consisted exclusively of tetraploid plants. Previously, Reis et al. (2008) analysed 53 accessions of *P. nicorae* from Rio Grande do Sul, Brazil, and all were tetraploid. Based on cytoembryological studies of ten tetraploid accessions of *P. nicorae*, Burson and Bennett (1970) considered that this species reproduced by obligate apomixis. However, our results show that a small degree of residual sexuality is detectable by flow cytometric analysis, and may play a significant role in the generation of genotypic variation within natural population of this species, in the same way as occurs in other agamic complexes with high quantitative effects over very short time scales (Hörandl and Paun 2007).

We observed a wide proportion of B_{III} in open-pollinated seeds from polyploid plants of *P. alcalinum*, *P. denticulatum*, *P. nicorae*, and *P. rufum*. Additionally, some hexaploids were recovered in a complementary screening among young plants established from open pollinated tetraploid plants of *P. nicorae* and *P. denticulatum* (data not shown). Considering only the tetraploid level of *P. denticulatum*, *P. nicorae*, and *P. rufum*, we observed the formation of B_{III} seeds in selected tetraploid plants of eight populations (D1, D2, D3, N1, N2, N3, R1, and R4). Curiously, not even a single hexaploid was discovered among the total of 538 tetraploid plants observed in these eight populations. Neopolyploids are successful only if they are able to compete successfully with their parents and other taxa for available habitats; this success depends on their fitness and their ability to overcome their minority status (de Wet 1980; Felber 1991). The lack of hexaploids among those tetraploid populations that usually form B_{III} seeds (and also seedlings) suggests that they may be selected against, possibly because of some fitness disadvantage. This disadvantage would not be caused by the concurrence of pseudogamy and the pollen of the wrong ploidy level that the newly formed hexaploids would receive from surrounding tetraploids. Apomictic pseudogamous *Paspalum* species are likely insensitive to pollen ploidy level as was established for the apomictic tetraploid *P. notatum* (Quarin 1999). Determination of the exact nature of this disadvantage would require further studies on the establishment of neopolyploids above tetraploid level and their persistence.

Paspalum alcalinum is a particular case with respect to the other species analysed in this study. Burson (1997) reported cytoembryological data concerning three accessions of this species from the Chaco region in Paraguay and one from central Argentina. He described one sexual diploid, one facultative apomictic tetraploid and one obligate apomictic pentaploid cytotype from Paraguay, while the accession from Argentina was a facultative tetraploid. However, we analysed four populations native to northern Argentina and Paraguay. The results showed that three of them produce exclusively hexaploid plants while the other was a mixed population with mainly pentaploid, some hexaploid and a rare heptaploid plant. All the plants selected to determine the method of reproduction proved to be obligate apomicts. No diploid or tetraploid cytotypes were identified even though one of the analysed populations was sampled in the same locality quoted by Burson (1997) for a tetraploid accession. For that reason, additional collection trips were performed, including ten localities from central Argentina, but again only 5x and 6x

plants were found (data not shown). Remarkably, hexaploid individuals of A2 and A3 populations also generated B_{III} seeds (9x) in open pollination but, as we indicated above for 4x populations of other species, the expected 9x plants were not found in the field. Similarly, in *Potentilla argentea*, enneaploids (9x) were not found when hexaploid populations were analysed, but they were recovered from seeds harvested in a greenhouse (Holm and Ghatnekar 1996).

These results emphasise the importance of studying the dynamics of genetic systems in natural populations of *Paspalum*, a genus in which agamic complexes play a decisive role in evolution and species formation. This approach allows a deeper understanding of the ploidy level/reproductive behaviour relationship and its evolutionary significance. The identification of novel diploid populations renders new materials for genetic improvement, since diploids are the reservoir of genetic variation and because induced autotetraploidy allows breaking of the apomictic barrier by crossing sexual induced neotetraploids with pollen of natural apomictic tetraploids. These findings also reveal the reproductive diversity of these species, ranging from complete sexuality in diploids, through varying levels of facultative apomixis in most tetraploids, to obligate apomixis in pentaploids and hexaploids. Our study also suggests that, in these sexual-diploid/apomictic-polyploid systems, fully sexual tetraploid plants do not occur in natural populations. However, several individuals with different origins presented residual sexuality, which could be a source of genetic variation in polyploid populations by generation of new apomictic genotypes.

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