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Cytogeography and reproduction of the *Paspalum simplex* polyploid complex

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Abstract. *Paspalum simplex* is a grass distributed throughout the phytogeographic Chaco region in South America from which sexual diploid and apomictic tetraploid races have been reported. We analysed native populations to determine their homogeneity of ploidy level, and the relationship between geographic distribution, ploidy levels, and reproductive systems. The ploidy level was established for 379 plants from 32 wild populations. Tetraploidy and apomixis constitute the most common combination for this species all over the Chaco region. Apomictic hexaploid plants were found associated with 4x populations. Diploids were confined to a small sector of the region. One sexual triploid plant arose from seed harvested in a pure 2x population, and one apomictic 3x plant was found in a mixed 2x-4x population. The results suggest that *P. simplex* is a core agamic complex characteristic of the Chaco region from which other apomictic polyploid species of the subgenus *Anachyris* could have evolved.

Key words: *Paspalum simplex*, agamic complex, apomixis, *Anachyris*, phytogeography.

Paspalum simplex Morong ex Britton is a warm season grass naturally distributed throughout the phytogeographic Chaco province in south-eastern Bolivia, western Paraguay and central-northern to north-eastern Argentina, stretching out the phytogeographic province up to eastern Paraguay, western border of central Brazil and north-western corner of Uruguay (Morrone et al. 2000). It is a frequent constituent of the native pasture grasslands and is considered an important species for domestication and plant improvement due to its forage potential. *Paspalum simplex* is one of those species of the genus for which sexual diploid ($2n = 2x = 20$) (Espinoza and Quarin 1997), as well as apomictic tetraploid races ($2n = 4x = 40$) have been reported (Nath et al. 1970, Caponio and Quarin 1987, Cáceres et al. 1999). Like in other species of the genus, possessing sexual-diploid and apomictic-tetraploid races, tetraploid plants of *P. simplex* with 100% sexual reproduction have never been found in the wild. However, 100% sexual tetraploid plants of *P. simplex* have been recovered experimentally by colchicine treatment of sexual diploid plants (Cáceres et al. 1999).

Meiotic chromosome behaviour in apomictic tetraploids suggests autoploidy as the most likely origin for these polyploid races (Nath et al. 1970, Caponio and Quarin 1987). Segregation analysis based on RFLP (restriction fragment length polymorphism) markers demonstrated the tetrasomic inheritance of *P. simplex* (Pupilli et al. 1997).

Our objective was to analyse the relationship between the geographic distribution and ploidy levels in this species. We wondered whether both sexual diploids and apomictic tetraploids are distributed all over the area of the species, whether any other ploidy levels exist for the species, and what were the patterns of coexistence among the different cytotypes.

Materials and methods

Thirty-two populations were investigated in a wide range of geographic distribution. Some accessions were collected by one small cutting with a short rhizome in the base from individual plants of wild populations. For some populations only one individual plant was available for chromosome counts because they belonged to already existing accessions in the active germplasm collection at Corrientes. For some localities two well separated populations were analysed. Cuttings were grown in pots in a greenhouse for chromosome counts. Other accessions were established from seeds harvested from approximately 50 individual plants in wild populations. Seeds of each accession were mixed and a sample was germinated in sterilised soil. Seedlings were transplanted in pots for further chromosome counts or for estimation of ploidy level by flow cytometry. A herbarium voucher specimen was collected in the field from each population. In addition, when a particular plant showed a chromosome number different from the normal ploidy level of a given population, the plant was grown in a greenhouse and a herbarium voucher was collected at flowering. The original vouchers were deposited in the CTES herbarium and duplicated specimens were distributed to several public European and American herbaria.

The chromosome number for each individual was determined either by counting the somatic chromosomes in root tips at Corrientes, Argentina, or estimated by flow cytometry at Campo Grande, MS, Brazil.

For chromosome counting, root tips were collected from potted plants, placed without fixation in a saturated solution of α-bromonaphthalene for 2 h, and hydrolysed in 1 N HCl for 10 min at 60 °C. Root tips were macerated in a drop of acetocarmine stain, heated, pressed under a cover-slip, and observed using a light transmission microscopy.

Ploidy levels were estimated by flow cytometry as an alternative for the root tip chromosome counting. The fluorescence intensity of DAPI-stained nuclei was determined using the flow cytometer Partec CA II (Partec GmbH, Münster, Germany). Approximately 0.5 cm^2 of young leaf tissue of the target plant was placed in a small Petri dish, together with a similar amount of leaf tissue of the control, a diploid plant of *P. simplex* determined by chromosome count in root tips. A drop of a nuclei extraction buffer (Partec CyStain UV solution A) was added and the tissue was chopped with a sharp razor blade. Immediately, the sample was adjusted to 0.5 ml with extraction buffer, the mixture was passed through a 30 μm filter directly into the sample tube, then 2 ml of Partec CyStain UV solution B (DAPI: 4′,6,diamidino-2-phenylindole) was added and the mixture was incubated for 5 min at room temperature and ran through the flow cytometer. The ploidy level was estimated in relation to the DNA peak of the check, a cytologically controlled diploid plant of *P. simplex*, used as an internal standard. In some accessions we counted the chromosome numbers at Corrientes and then some plants were taken to Campo Grande for flow cytometry analysis. Otherwise, some accessions were germinated at Campo Grande, analysed by flow cytometry, and then a few plants of those accessions were brought to Corrientes to corroborate the ploidy levels by chromosome counting in root tips.

The general geographic distribution of the species was outlined according to Morrone et al. (2000) and the data obtained during our field collection trips and herbarium work. In addition to our own data concerning chromosome numbers, the few previous counts from literature were taken into account to outline the specific distribution limits of each chromosome race.

Embryological analyses were conducted in order to study the method of reproduction of some plants which had ploidy levels different from the already known diploids or tetraploids. Spikelets at anthesis were fixed overnight in FAA (70% ethanol – glacial acetic acid – 37% formaldehyde, 18:1:1), ovaries were dissected, cleared with Herr's technique (1971) and observed using differential interferential contrast microscopy. Plants bearing a single embryo sac per ovule typically organised with an egg apparatus, a large two-nucleate central cell and several antipodal cells, were considered to reproduce by sexual means. Plants in which at least some ovules had multiple embryo sacs lacking antipodal cells, differing in size, orientation and cellular organisation, were recorded as apomictics due to their capability of aposporous embryo sac formation. No discrimination was done concerning the degree of apomictic reproduction for each individual plant. Though facultative apomixis occurs in many *Paspalum* species, the degree of sexual reproduction in any apomictic individual is generally low. We stressed the genetic capability for apomictic reproduction against the incapability to produce aposporous embryo sacs. An individual plant is considered to be sexual when only meiotic embryo sacs are formed in its ovules, while an apomictic plant forms one or more aposporous embryo sacs per ovule, though occasionally some ovules may contain a meiotic embryo sac. Completely sexual tetraploid individuals have never been found in natural populations of apomictic tetraploid *P. simplex*.

Results

From the overall chromosome counting and ploidy level estimation, it was established that tetraploidy was the most common ploidy level for this species (Table 1, Fig. 1). In addition to the previously recognised sexual diploids and apomictic tetraploids, two triploid plants and several hexaploids were recorded. Hexaploid plants were associated with some tetraploid populations while one out of 28 seedlings of the diploid accession U-14 proved to be triploid by flow cytometry, and confirmed by chromosome counting ($2n = 3x = 30$). Only one single and typical embryo sac per ovule was observed, suggesting sexual reproduction for this triploid plant. A second triploid was detected by chromosome counting among a mixed 2x-4x population. In this case, multiple embryo sacs observed in many ovules suggested apomictic reproduction. Multiple embryo sac development, observed in several ovules of three 6x plants from different accessions, indicated that apomixis is the method of reproduction at the hexaploid level.

Most native populations were tetraploid and extended over the entire distribution range of the species. Usually, diploids and tetraploids constituted homogeneous populations concerning ploidy level. Only one mixed 2x-4x population was recorded at the border of diploid distribution area. Hexaploid plants were invariably associated with tetraploid populations and pure hexaploid populations have not been observed. Diploids were confined to the western sector of the general distribution range of the species (Fig. 1).

Morphologically, diploid, triploid, tetraploid and hexaploid plants were very similar and we were unable to identify any exomorphological character specific of some ploidy level.

Discussion

Because tetraploids are apomictic, and hexaploid plants invariably inhabited 4x populations, it is suggested that hexaploids could arise through $2n + n$ fertilisation. In apomictic grasses the unreduced female gamete can be eventually fertilised by a normal reduced male gamete, due to the failure of parthenogenesis in an aposporous embryo sac.

Triploids seem to be rare individuals which arise among a diploid population, probably through unreduced gametes ($2n + n$) or in mixed 2x-4x populations, where there are two possible origins: by unreduced gametes as in diploid populations, or by 2x × 4x crosses. Theoretically, the origin is more likely by 2x × 4x crosses; however, experimental crosses in other species of *Paspalum* demonstrated that post-zygotic seed abortion impedes the production of triploid hybrids after 2x × 4x co-specific crosses, or reduces the chances to less than 0.03% (Norrmann et al. 1994).

Table 1. Ploidy levels observed among wild populations of *Paspalum simplex*, using either flow cytometry estimation (**FC**), chromosome counting (**CC**) or both (**FC + CC**). The abbreviations Ch, C, F, Sf and Sg stand for the following provinces of Argentina: Chaco, Corrientes, Formosa, Santa Fe and Santiago del Estero, respectively. Different populations from the same locality are indicated (A) and (B)

Accession	Locality of collection	Number of plants analysed	Number of individuals studied by			Number of individuals with			
			CC	FC	FC + CC	2X	3X	4X	6X
U-8	Makallé, Ch	12	1	9	2			12	
U-9	Quitilipi, Ch	18	1	15	2			17	1
U-14	Aviá-Terai, Ch	28	11	14	3	27	1		
U-17	Charata, Ch	13	1	10	2	13			
U-18	Cabaña Los Gatos, Sg	22	–	19	3	22			
U-25	Gancedo (A), Ch	15	1	12	2	15			
U-26	Gancedo (B), Ch	20	–	17	3	20			
U-27	Pinedo, Ch	14	1	11	2	14			
U-28	Santa Sylvina, Ch	14	2	10	2	14			
U-29	Villa Ángela, Ch	86	70	14	2	63	1	22	
U-30	P. Plaza, Ch	6	–	3	3			6	
U-31	Reconquista, Sf	12	1	9	2			12	
U-32	Paso de la Patria (A), C	8	–	5	3			8	
U-33	Calchaquí, Sf	10	–	10	–			10	
U-34	Basaíl, Ch	13	–	13	–			13	
U-35	Saladas, C	1	1	–	–			1	
U-53	J.J. Castelli, Ch	2	2	–	–			2	
Q-4114	City of Corrientes (A)	1	1	–	–			1	
Q-4116	City of Corrientes (B)	1	1	–	–			1	
Q-4121	Alejandra, Sf	3	3	–	–			3	
Q-4124	Villa Ana, Sf	2	2	–	–			2	
Q-4129	Paso de la Patria (B), C	2	2	–	–			2	
Q-4146	San Roque, C	5	1	2	2			4	1
Q-4168	Mercedes, C	13	–	7	6			12	1
Q-4189	Pirane (A), F	16	–	12	4			10	6
Q-4190	Pirané (B), F	13	1	10	2			13	
Q-4191	Palo Santo (A), F	2	2	–	–			2	
Q-4195	Palo Santo (B), F	3	–	1	2			3	
Q-4197	Pozo del Tigre, F	4	–	3	1			4	
Q-4200	Ibarreta, F	14	2	11	1			13	1
BRA-007480A	Porto Mourtiño, Brasil	1	1	–	–			1	
**ST-13817	Prov. L. Calvo, Bolivia	5	5	–	–			5	
	Total	379	113	217	49	188	2	179	10

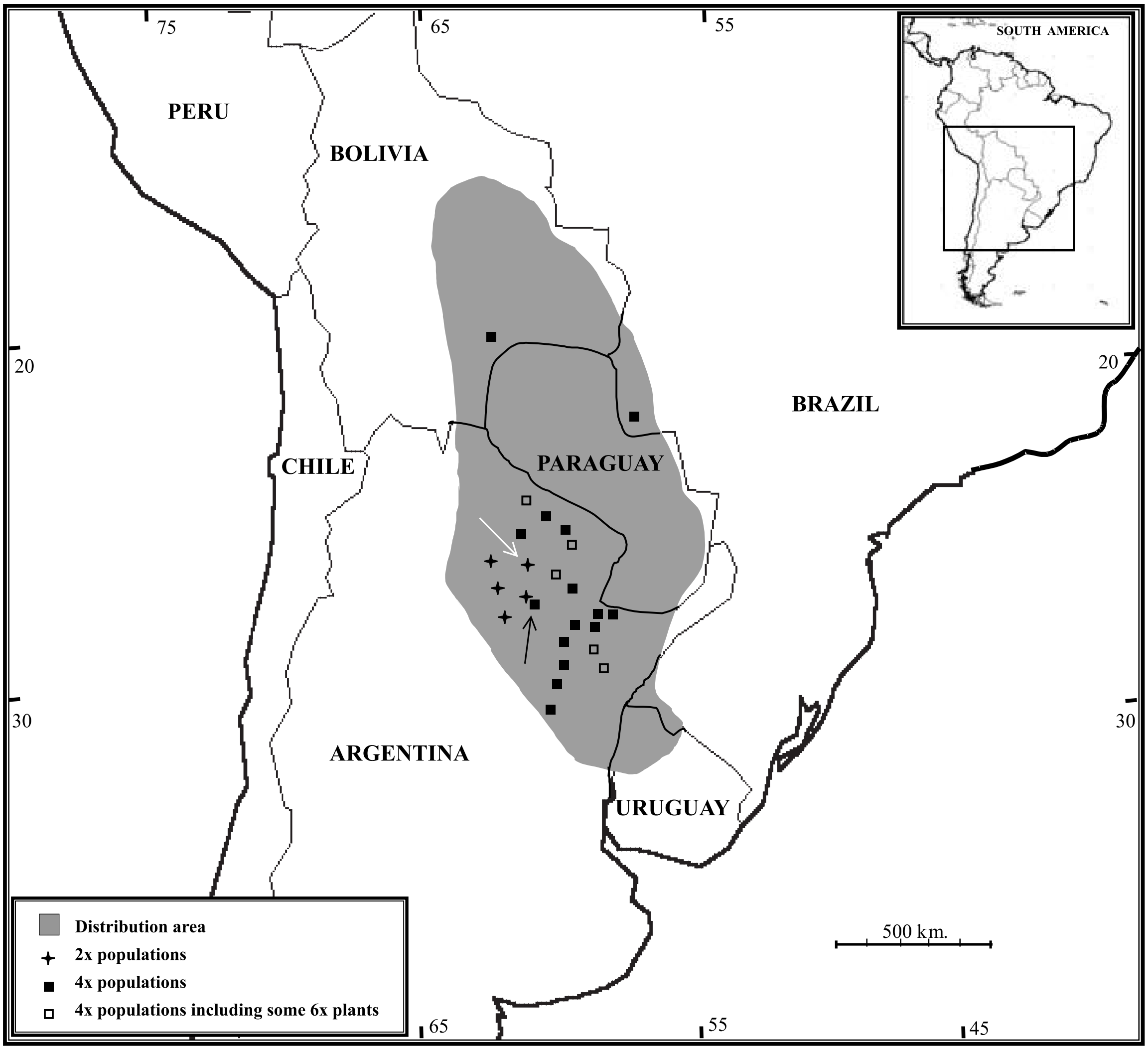

Fig. 1. Distribution area of *Paspalum simplex* and ploidy levels of several of the analysed populations. The arrows point to the places where individual triploid plants were found: one sexual 3x plant in a 2x population (white arrow), and the apomictic 3x plant in a mixed 2x-4x population (black arrow)

The phytogeographic Chaco province, as it was outlined by Cabrera and Willink (1973), constitutes of vast plains in South America from south-eastern Bolivia and western Paraguay to central-northern and north-eastern Argentina. The vegetation is mostly xerophytic woodlands or, in some areas, savannas (Cabrera and Willink 1973). The annual rainfall increases from 500 mm in the north-west corner of the region to 1,300 mm in the eastern part, near the Paraguay and Paraná rivers (Glatzle 1999). *Paspalum simplex* is distributed all over this region, even exceeding the south-eastern limits of the region in the provinces of Corrientes and Entre Ríos, Argentina, up to the north-western corner of Uruguay. Diploids are confined in the central west part of this region, in the driest and less favourable climatic areas, below the Tropic of Capricorn. This distribution pattern is clearly contrasting with the well known cytogeography of the polymorphic grass *Themeda triandra* in South Africa as described by de Wet (1960). The diploid races of *Th. triandra* are present throughout the range of the species but are most abundant in the more favourable climatic areas. The percentage of diploids drops and polyploids take over toward the cooler areas and dry regions. In *P. simplex* diploids are confined to one of the less favourable climatic area within the range of the species.

Paspalum simplex belongs to the subgenus *Anachyris*, for which modern authors recognise only 6 species (Renvoize et al. 1998, Morrone et al. 2000). *Paspalum simplex* and *P. procurrens* Quarin are native and characteristic of the Chaco phytogeographic province, and coincidentally these are the two species of the subgenus for which 2x cytotypes have been mentioned (Quarin 1993, Espinoza and Quarin 1997). Morphologically, diploid strains of *P. simplex* and *P. procurrens* are easily recognised as different species, yet the high crossability and the high level of fertility observed in their controlled interspecific hybrids and backcrossed progenies, contrast with the lack of intermediate forms observed either in herbaria or in the field, suggesting that divergent evolution occurred in correlation with latitude and specific soil requirements (Espinoza and Quarin 1998). The other four species of the subgenus *Anachyris* are distributed outside the Chaco phytogeographic province or may be found in the borders of the region, as is the case of *P. malacophyllum* Trin., an autotetraploid species (Burson and Hussey 1998), native of Mexico and South America where it distributed in upland savannas, open slopes and cliffs, from sea level up to 3,000 m altitude in Brazil, Paraguay, Bolivia and central west and northern Argentina. However, it is absent from the vast central area of the phytogeographic Chaco province (Morrone et al. 2000). *Paspalum usteri* Hack. is a tetraploid (Hunziker et al. 1998) and is distributed from central eastern Brazil to eastern Paraguay and north-eastern Argentina, *P. volcanensis* Zuloaga, Morrone & Denham grows in the Yungas phytogeographic province and is tetraploid (Hunziker et al. 1998, Zuloaga et al. 2000, Morrone et al. 2000), and *P. constellatum* Swallen from northern Brazil, for which no information is available regarding chromosome number and reproductive mode. *Anachyris* is a well characterised subgenus with a typical spikelet morphology that makes it distinguishable from any other *Paspalum* species (Morrone et al. 2000). This peculiar spikelet morph that embraces the six recognised taxa, and the autoploid origin and apomictic reproduction observed in some species (*P. simplex*, *P. malacophyllum* and likely *P. volcanensis*), suggest that the whole subgenus could be an important agamic complex. The different morphological entities of this complex, actually recognised as separate species, may have evolved and differentiated at the diploid level. Diploid strains of *P. simplex* and *P. procurrens* have small distribution areas close to each other. They are sexual and allogamous due to self-incompatibility (Quarin 1993, Espinoza and Quarin 1997) and may well be the ancestors from which all tetraploid species of *Anachyris* have evolved through autoploidy, apomixis and eventual sexual reproduction. Alternatively it may be hypothesised that the actually existing species evolved from already differentiated diploid species, most of which disappeared or are so rare that they have not been collected. The hypothesis of species divergence at the diploid level is supported by the close morphological similarities between 2x and 4x races either in *P. simplex* or in *P. procurrens* (a 4x race of *P. procurrens* has been found recently, Schegg and Quarin, unpublished). There are sharp morphological differences between 2x *P. simplex* and 2x *P. procurrens* and these differences are maintained at the 4x level. Diploid races of *P. malacophyllum*, *P. volcanensis*, or *P. usteri* have not been collected so far, and the ploidy level of *P. constellatum* is still unknown.

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