

## Sexuality, apomixis, ploidy levels, and genomic relationships among four *Paspalum* species of the subgenus *Anachyris* (Poaceae)

Diego Hojsgaard<sup>a</sup>, Esteban Schegg<sup>a</sup>, José F.M. Valls<sup>b</sup>, Eric J. Martínez<sup>a</sup>,  
Camilo L. Quarín<sup>a,\*</sup>

<sup>a</sup>Facultad de Ciencias Agrarias, Instituto de Botánica del Nordeste (IBONE), Universidad Nacional del Nordeste (UNNE), Casilla de Correo 209, 3400 Corrientes, Argentina

<sup>b</sup>Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia (EMBRAPA-CENARGEN), SAIN – PqEB, Caixa Postal 02372, 70770-900 Brasília, DF, Brazil

Received 17 May 2007; accepted 27 September 2007

### Abstract

The subgenus *Anachyris* of the genus *Paspalum* comprises six species, all native to the New World. Cytology, embryology, fertility, and hybridization experiments were examined in four of the six species and different chromosome races. Meiotic chromosome behavior supported autopolyploid origin for tetraploid *P. procurrens* and tetraploid *P. usterii*, and also corroborates autopolyploidy for *P. malacophyllum*. Meiosis of the three species was compared with the meiotic chromosome behavior of a tetraploid *P. simplex* strain acquired by artificial autopolyploidization. Moreover, chromosome associations observed in tetraploid interspecific hybrids of *P. simplex* × *procurrens* and *P. simplex* × *usterii*, in triploid hybrids of *P. simplex* × *malacophyllum*, and in diploid hybrids of *P. simplex* × *malacophyllum*, indicate that the four species share the same basic genome, and that the polyploid races are autopolyploids. Facultative apomixis of the aposporous type is reported for the first time in tetraploid strains of *P. procurrens* and *P. usterii*. It is also confirmed for tetraploid *P. malacophyllum*, a species whose diploid representative is here reported to reproduce sexually, though embryological analyses demonstrated some potential for apospory. Our results suggest that the whole subgenus *Anachyris* of *Paspalum* is basically an agamic complex. At least three species: *P. simplex*, *P. procurrens*, and *P. malacophyllum* involve sexual self-incompatible diploid races as well as apomictic polyploid (mainly tetraploid) representatives.

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**Keywords:** *Paspalum*; *Anachyris*; Autopolyploidy; Interspecific hybrids

### Introduction

The subgenus *Anachyris* of the genus *Paspalum* includes six species according to the taxonomic revision of Morrone et al. (2000). The subgenus is distributed from Mexico to Brazil, Paraguay, Bolivia, Argentina

and Uruguay. The main distinctive morphological characteristics are: the boat-shaped, concave–convex spikelets; the second glume typically missing, though occasionally and incompletely developed in two species; and the upper lemma with prominent longitudinal nerves on the abaxial surface.

In the genus *Paspalum*, most species are characterized by the existence of different conspecific cytotypes. Usually, the diploid strains reproduce by sexuality and

\*Corresponding author.

E-mail address: [quarin@agr.unne.edu.ar](mailto:quarin@agr.unne.edu.ar) (C.L. Quarín).

cross-fertilization, due to self-incompatibility. The conspecific polyploid strains, usually tetraploids, reproduce by means of aposporous apomixis (Quarin, 1992). Among the species of the subgenus *Anachyris*, *P. simplex* Morong is a good example of this genetic system: the sexual cross-pollinating diploid cytotype has a limited distribution area in central-northern Argentina, while the apomictic tetraploid cytotype has a broader distribution including areas of Bolivia, Paraguay, Brazil, Argentina and Uruguay. Two uncommon triploid plants have been collected. One of these was sexual and the other was apomictic. In addition, hexaploid apomictic plants have occasionally been found accompanying tetraploid populations (Urbani et al., 2002). Sexual self-incompatible diploid accessions of *P. procurrens* Quarin are known to be geographically congruent and cytologically related to diploid *P. simplex* (Espinoza and Quarin, 1998). Until now, polyploid accessions have not been reported for *P. procurrens*.

*P. malacophyllum* Trin. is the most widely distributed species and shows the greatest morphological variation among the species (Morrone et al., 2000) of *Anachyris*. The literature contains conflicting reports concerning the mode of reproduction of this species, though all studies indicate that the species is tetraploid. It was first reported to be an aposporous apomict by Brown and Emery (1958). Later, Bennett and Bashaw (1966) indicated that the species was sexual and autotetraploid. Based on cytoembryological studies of 16 accessions collected in different locations of Argentina and Brazil, Burson and Hussey (1998) considered *P. malacophyllum* to be an autotetraploid, with the mode of reproduction being facultative apomixis. All the analyzed accessions showed aposporous embryo sac development, though some meiotically derived embryo sacs were observed in all of them to different degrees of development. Recently, rare diploid accessions of *P. malacophyllum* were found in southern Brazil (Pozzobon, M., personal communication).

Limited information exists concerning ploidy level and mode of reproduction of the remaining three species of the subgenus *Anachyris*. *Paspalum usterii* Hackel and *P. volcanensis* Zuloaga, Morrone & Denham (sub *Paspalum* aff. *malacophyllum*) were reported to be tetraploid (Hunziker et al., 1998). There is no information concerning the reproductive mode of these two species and of the ploidy level and reproduction of *P. costellatum* Swallen.

Taking advantage of the existence of sexual diploid genotypes in *P. simplex* and *P. procurrens*, Espinoza and Quarin (1998) hybridized these species. The high level of cross-fertilization (66%), the regular meiotic chromosome pairing in the F<sub>1</sub> hybrids, and the proportion of spikelets that set seeds in the hybrids (62%) suggested a very close evolutionary relationship between these species.

Because all known tetraploid strains of the subgenus *Anachyris* are apomictic or highly apomictic, it would be difficult to study the genomic relationship among its species with interspecific crosses. The acquisition of sexual tetraploid plants of *P. simplex* from colchicine treatment of diploid plants (Cáceres et al., 1999) bypassed the difficulty of hybridization. The induced tetraploid *P. simplex* reproduces sexually and could be emasculated and pollinated with pollen of the other apomictic species of the subgenus.

The aims of this investigation were: (1) to report the occurrence of tetraploidy in *P. procurrens*; (2) to determine the meiotic chromosome behavior and the mode of reproduction of tetraploid accessions of *P. usterii* and *P. procurrens*, and of a diploid and two newly acquired tetraploid germoplasm accessions of *P. malacophyllum*; (3) to make interspecific crosses between a sexual, colchicine-induced tetraploid plant of *P. simplex* and three tetraploid species of the subgenus *Anachyris*: *P. malacophyllum*, *P. procurrens*, and *P. usterii* in order to determine their genomic relationships and the feasibility of introgression of genes among these species via hybridization programs; (4) to hybridize diploid *P. simplex* and the recently acquired diploid germoplasm of *P. malacophyllum* to assess the degree of homology of their genomes and the level of fertility of their hybrids.

## Material and methods

### Plant materials

The sexual diploid accession of *P. simplex* Q4109 was originally collected at Los Gatos ranch, Santiago del Estero, Argentina. The individual plant no. 59 was used as female parent in crosses with diploid *P. malacophyllum*.

Cuttings of an original colchicine-induced tetraploid plant of *P. simplex* were introduced to Argentina from Italy. This sexually reproducing tetraploid plant was experimentally produced at the Institute of Plant Genetics, CNR Research Division of Perugia (Cáceres et al., 1999). The plant was indeed a mixoploid with diploid and tetraploid sectors, but predominantly tetraploid. It was identified as 11B1a, multiplied vegetatively, and grown in pots in a greenhouse at IBONE, Corrientes. Most crosses were carried out using plant 11B1a as the female parent in interspecific crosses. The exception was the *P. simplex* × *usterii* cross for which the plant named C1-2 of *P. simplex* was used as the female parent. Plant C1-2 is a sexual tetraploid that originated from self-pollination of plant 11B1a.

*P. procurrens*, accession Q4094, is a newly acquired germoplasm collected at El Salvador, Chuquisaca,

Bolivia, by C. Saravia-Toledo, and grown from seeds in Corrientes, Argentina, by routine cultural practices.

Two 4x accessions of *P. malacophyllum* were used for interspecific crosses with induced 4x *P. simplex*: accession TK2449, collected by Tim Killeen 2 km N of Concepción, Estancia El Recreo, Santa Cruz, Bolivia (16°12'S, 62°08'W), and grown from seed at Corrientes. It was previously reported to be tetraploid (Norrman et al., 1994) and represents the typical morphology of the species, very similar to the drawings published with the original description by Trinius (1831). The second accession, V5095, was collected by José F. Valls, on the road between Itumbiara and Bom Jesus de Goiás, Brazil, and was reported to be tetraploid (Honfi et al., 1990). Then Pagliarini et al. (2001) studied the meiotic behavior of this same accession, under its Brazilian germplasm code BRA-003077, and found an average of 0.86 univalent, 10.34 bivalent, 0.48 trivalent and 4.05 quadrivalent chromosomes per cell. It is an atypical plant with short and extremely broad leaf blades (usually more than 4 cm wide). The plant was introduced as cuttings to Corrientes, Argentina in the early 1990s and maintained in the field by routine cultural practices. Unlike the accession TK2449 that represents the morphological archetype of the species, the plant V5095 represents an extreme degree of variation regarding the shape and size of leaf blades. It might well be considered a different taxon due to these morphological dissimilarities and its decumbent growth habit.

*P. usterii* was collected by Ana Honfi, accession 1175, at Teyucuaré, Misiones, Argentina, and cultivated from pieces of rhizomes in Corrientes, Argentina.

The diploid accession V14855 of *P. malacophyllum* was collected 7 km north of Correia Pinto, state of Santa Catarina, Brazil (27°31'48.3"S, 50°22'11.7"W). The growth habit and the leaf shape of this diploid plant resemble the tetraploid accession V5095, but the size of the whole plant and particularly the leaves is much smaller.

Vouchers of the studied material are deposited in the CTES herbarium at Corrientes, Argentina, with exception of *P. malacophyllum*, accessions V5095 and V14855, which are deposited in the CEN herbarium at Brasilia, Brazil.

### Interspecific hybridizations

The female parent, a potted plant of 11B1a, was introduced into a special humidity chamber late in the evening. Only one inflorescence, ready to start blooming the next day, was left on the plant. Early in the morning, a programmed humidifier raised the relative humidity in the chamber to about 100%. At blooming time, soon after sunrise, the anthers remained indehiscent after

anthesis due to the high humidity in the chamber. The hanging indehiscent anthers were eliminated with thin-pointed tweezers. Then, pollen of the desired male parent was harvested in glassine bags and dusted on the emasculated inflorescence. The plant was returned to the greenhouse with the inflorescence bagged in a glassine bag to prevent later undesired pollination. The whole procedure was repeated for 5–6 days until all the spikelets of the target inflorescence completed blooming and were pollinated. Approximately 30 days later, the inflorescence was harvested, the fertile spikelets were separated from the empty ones and sown in sterilized soil the following spring. Seedlings were separately grown in 200 cm<sup>3</sup> pots and then transplanted to the field. The same procedure was followed when the tetraploid plant C1-2 was used as female parent.

To cross 2x accessions of *P. simplex* and *P. malacophyllum*, a self-incompatible single plant of 2x *P. simplex*, accession Q4109, was isolated and daily dusted with pollen of accession V14855 of diploid *P. malacophyllum* until the target inflorescence completed flowering.

### Hybrid identification and crossability

Because the emasculation procedure might allow some minimal degree of self-pollination, and although the female parents produced few seed under self-pollination conditions, the progenies were controlled to ensure that each descendant was of hybrid origin. The recognition of the hybrids in the progeny of *P. simplex* × *procurrens* crosses was accomplished by observation of the growth habit: the female parent, *P. simplex*, has an erect habit while *P. procurrens* is stoloniferous, a dominant character. Thus, the occurrence of stoloniferous branches in the offspring was an indicator of hybridity. The hybrid origin among tetraploid individuals of the *P. simplex* × *malacophyllum* was estimated by morphological characteristics because there are clear differences between the parental species. Because the morphological characteristics of *P. simplex* and *P. usterii* are less contrasting than in the interspecific crosses mentioned above, the recognition of the hybrid origin of the progeny was conducted by molecular marker analysis: Specific bands generated by randomly amplified polymorphic DNA markers (RAPD) in the male parent were considered indicative of hybridization when the bands were observed in any individual plant of the progeny. The DNA extraction and RAPD procedures were as described in Acuña et al. (2004).

The whole progeny of the 2x *P. simplex* (female) × 2x *P. malacophyllum* (male) cross was assumed to be hybrids since the female progenitor was a self-incompatible plant, and because most morphological characteristics of the progeny was intermediate between parents.

The degree of crossability among the different species was calculated as the number of hybrids recovered for every hundred pollinated spikelets. In *Paspalum*, each spikelet bears a single perfect floret and the ovary supports a single ovule.

## Cytology

Mitotic chromosome numbers were determined in root tips, collected from potted plants, pretreated in a saturated aqueous solution of  $\alpha$ -bromonaphthalene for 2 h, hydrolyzed with 1 N HCl for 10 min at 60 °C, stained with Feulgen's reagent and squashed in a drop of 2% acetic orcein.

Inflorescences of parental plants and of selected hybrids were fixed in 3:1 solution of absolute ethanol–glacial acetic acid and stored in 70% ethanol at 5 °C. Pollen mother cells (PMC) for the study of meiotic chromosome behavior were stained with 2% acetocarmine.

## Mode of reproduction

The mode of reproduction of tetraploid *P. procurrens* and of two 4x accessions of *P. malacophyllum* was determined by the study of megasporogenesis and embryo sac development. Ovaries at different stages of development were fixed in FAA (10% ethanol, glacial acetic acid, and 37% formaldehyde in the ratio 18:1:1), dehydrated in a tertiary butyl alcohol series, and embedded in paraffin. Material was sectioned 12  $\mu$ m thick, stained in safranin–fast green staining series, and observed under bright-field microscopy. The proportion of ovules carrying aposporous or meiotic embryo sacs was determined in ovaries fixed at the time of anthesis. Only mature ovaries, fixed at the time of anthesis, were sectioned, stained, and analyzed to assess the mode of reproduction of *P. usterii* and the diploid accession V14855 of *P. malacophyllum*.

## Fertility

In most species of *Paspalum*, including all species of the subgenus *Anachyris*, each spikelet contains a single perfect flower bearing a single ovule in its ovary. The degree of fertility was assessed as the proportion of spikelets that formed a mature caryopsis. Fertility was determined under self-pollination conditions, open-pollination, and by backcrossing. Self-pollination was achieved by bagging whole inflorescences with glassine bags to enforce self-pollination and prevent outcrossing. Open pollination was accomplished by harvesting mature inflorescences from a plant that flowered among other genotypes of the same species. For interspecific hybrids, open pollination fertility was analyzed in

mature inflorescences of some selected plants of the hybrid population cultivated in the field without any control of pollination. Backcrossing was achieved by supplying parental pollen to some selected inflorescences of the hybrids.

## Results

### The ploidy of *P. procurrens* and *P. usterii*

The accession Q4094 of *P. procurrens* was tetraploid with  $2n = 40$  chromosomes. Counts were performed on root tips of several plants obtained from seed of the original collection. The accession H1175 of *P. usterii* was established by cuttings of the original collection and had  $2n = 40$  chromosomes.

### Reproductive mode in male parental species

Eighty-six mature ovules were observed in the diploid accession V14855 of *P. malacophyllum*. Most ovules (89.6%) showed a single embryo sac of the meiotic type (Table 1) with the egg apparatus (two synergids and the egg) toward the micropylar end, a large oblong and highly vacuolated central cell with two polar nuclei, and a mass of proliferated antipodal cells at the chalazal end. This is the typical feature of the embryo sac in sexually reproducing grass species. Occasionally, an aposporous embryo sac, bearing an egg cell and a central cell with two polar nuclei, was observed beside the typical meiotic sac and sharing the same ovule (4.6%), while 5.8% of the mature ovules failed to develop an embryo sac.

The reproductive mode was determined by the examination of megasporogenesis, megagametogenesis, and mature embryo sacs in tetraploid accessions of *P. malacophyllum* and *P. procurrens*. A conspicuously elongated megasporocyte was observed in young ovules of *P. malacophyllum* TK2449, before the inner integument completed development and before the micropyle was formed toward the base of the ovary. Meiosis took place during ovule rotation. The first cycle of meiosis and cytokinesis resulted in the formation of two dyad cells. The second division and cytokinesis took place in the chalazal member of the dyad and formed a final linear triad of megaspores with the simultaneous arrest of the second meiotic cycle in the micropylar dyad. The central megaspore degenerated immediately, followed by the abortion of the micropylar megaspore. The residue of this megaspore surrounded by a thick layer of callose remained as a dark and dense body in the micropylar end of the nucellus. By the time the ovule had finished rotation and the inner integument was complete (the outer integument was arrested in development and failed to cover the whole ovule), both

**Table 1.** Embryo sac types in mature ovules of different *Paspalum* species and accessions

Species	$2n$	Number of ovules observed	Percentage of ovules with			
			One meiotic sac	One meiotic + one to several aposporous sacs	One to several aposporous sacs	Deteriorated sacs
<i>P. malacophyllum</i> V14855	20	86	89.6	4.6	0.0	5.8
<i>P. malacophyllum</i> TK2449	40	67	3.0	4.5	79.1	13.4
<i>P. malacophyllum</i> V5095	40	76	17.1	7.9	64.5	10.5
<i>P. procurrens</i> Q4094	40	72	27.8	36.1	33.3	2.8
<i>P. usterii</i> H1175	40	79	35.4	13.9	41.8	8.9

megaspores closest to the micropyle had already degenerated. The chalazal megaspore remained as the functional megaspore in 10 out of 17 ovules analyzed at this stage, while in the other seven ovules all three megaspores aborted. Active nucellar cells (initials of aposporous embryo sacs) with dark cytoplasm and a conspicuously enlarged nucleolus were observed close to the degenerated megaspores. Usually, when the chalazal megaspore remained functional it was also accompanied by one to several active nucellar cells. Thus, the majority of the ovules showed initials of aposporous embryo sacs at the end of megasporogenesis regardless of whether the chalazal megaspore remained functional or not. Aposporous embryo sacs developed from these initials. At maturity, the aposporous sacs organized with an egg cell, one or two synergids (occasionally no synergids were observed), a large central cell with two (eventually three) polar nuclei, and usually an absence of antipodal cells. The number of aposporous sacs observed per mature ovule was variable, though usually only one or two became fully developed at maturity. When the chalazal megaspore escaped abortion it developed into a meiotic embryo sac, distinguishable by the proliferation of antipodal cells. At maturity, embryo sacs with few antipodal cells (3–6) were occasionally observed. Although these sacs might be aposporous sacs of the *Hieracium* type, we considered that all sacs bearing antipodal cells were meiotic embryo sacs. If this judgment is incorrect, the degree of sexuality would be somewhat overestimated. Proembryos were frequently observed in aposporous embryo sacs by the time of anthesis, prior to pollination. In a limited number of ovules, no female gametophyte developed. Megasporogenesis and embryo sac development of *P. malacophyllum* V5095 and *P. procurrens* Q4094 followed a similar patterns to the ones described above for *P. malacophyllum* TK2449. The main differences were that in *P. malacophyllum* V5095 usually female meiosis gave rise to four megaspores. Frequently, the four megaspores degenerated. A normal functional megaspore was observed in less than 10% of the ovules analyzed at the end of female meiosis. Development of proembryos by the time of anthesis, in aposporous

embryo sacs, was observed less frequently in *P. malacophyllum* V5095 and in *P. procurrens* Q4094 than in *P. malacophyllum* TK2449.

In summary, four developmental classes of ovules were observed at maturity in all the tetraploid species or strains studied: (1) with one meiotic embryo sac; (2) with the meiotic embryo sac plus one or more aposporous sacs; (3) with one to several aposporous sacs; or (4) aborted ovules. The proportion of mature ovaries (fixed at anthesis) observed with these four classes of ovules in *P. malacophyllum* TK2449, *P. malacophyllum* V5095, *P. procurrens* Q4094 and *P. usterii* H1175 is shown in Table 1. It is considered that mature ovules bearing one or more aposporous sacs would lead to the formation of seed through parthenogenesis of the unreduced ( $2n$ ) egg cell, and hence by mean of apomixis. On the other hand, those ovules carrying a meiotic embryo sac would need fertilization of the haploid ( $n$ ) egg cell to form the embryo of a sexually formed seed. Those ovules carrying aposporous sacs together with the normal meiotic sac would give rise to seeds that may contain either a sexually formed embryo or a parthenogenic embryo, or both in the same seed. Although polyembryonic seeds are possible, germination of multiple seedlings from one seed was not observed in this material.

The reproductive mode of tetraploid *P. usterii* H1175 was assessed by the analysis of embryo sac types in mature ovules fixed at the time of anthesis. Nearly 42% of the ovules showed embryo sacs of the aposporous type (Table 1). In these ovules, usually one or two well-developed embryo sacs were present while some underdeveloped sacs were sporadically observed. Over 35% of the ovules had a single embryo sac characterized by an egg cell and two synergids toward the micropylar end, a large, highly vacuolated central cell with two nuclei (occasionally three), and three, rarely four, antipodal cells at the chalazal end. These antipodals showed mostly vacuolated and transparent cytoplasm with an inconspicuous or collapsed nucleus at the time of anthesis. We classified these sacs as meiotic embryo sacs because the development of antipodal cells, in the grass family, is a typical feature of embryo sacs that develop from the functional megaspore after meiosis.

Less frequently, some ovules (13.9%) showed a meiotic sac flanked by one or two aposporous sacs, and mature ovules lacking embryo sac were also observed (8.9%).

## Interspecific hybridization

### *Paspalum simplex* 11B1a × *procurrens* Q4094

From 542 spikelets of sexual-induced tetraploid *P. simplex*, which were emasculated and dusted with pollen of tetraploid *P. procurrens*, 92 caryopses were formed, and 74 seedlings were recovered after germination. Two seedlings died and 72 were transplanted to the field. Four adult plants showed the typical upright phenotype of the female parent and were considered to have originated from self-pollination of the female parent due to occasional failure in the emasculation process. The remaining 68 plants were classified as hybrids because all of them developed stoloniferous branches, which is a characteristic of the male parent, *P. procurrens*. Thus, the effective crossability between *P. simplex* and *P. procurrens* was 12.5% at the tetraploid level.

### *Paspalum simplex* 11B1a × *malacophyllum* TK2449

One hundred and sixty-eight spikelets of a single *P. simplex* inflorescence were emasculated and pollinated with pollen of accession TK2449. Twenty-six spikelets formed caryopses and 22 seedlings were recovered after germination. The seedlings were transplanted to the field. Selected phenotypic characteristics were analyzed and compared with the parental traits. In all 22 progenies, most of the traits assessed were intermediate to those of the parents or closer to the values observed in the male than in the female parent (Table 2). Thus, the general phenotype of the whole progeny left little doubt about the hybrid origin of all recovered descendants. Thus, the crossability between

sexual tetraploid *P. simplex* and the tetraploid accession TK2449 of *P. malacophyllum* was 13.1%.

### *Paspalum simplex* 11B1a × *malacophyllum* V5095

Crosses were made on a single inflorescence of 11B1a (female parent). Only 228 spikelets were emasculated and dusted with pollen of V5095 and the remaining spikelets were removed from the inflorescence before blooming. Although 19 caryopses were formed, only nine seedlings were recovered after germination. Since there was a clear difference in morphology among parental plants, mainly in the size and shape of their leaf blades, the nine plants were easily classified as hybrids. All of them showed prominently broad leaf blades, a characteristic of the V5095 accession. The nine plants were cultivated in the field and showed very broad leaf blades ranging between 1.7 and 2.9 cm in width with a mean of 2.4 cm, while the mean leaf blade width for the 11B1a female parent was 0.9 cm, and 4.4 cm for the male parent V5095. Because the nine plants were classified as hybrids, the crossability was 3.78%.

### *Paspalum simplex* C1-2 × *usterii* H1175

The crosses were conducted using the tetraploid plant C1-2 of *P. simplex* as female parent. Plant C1-2 in turn had been obtained by selfing the 11B1a plant. The pollinator was the tetraploid accession H1175 of *P. usterii*. A total of 2200 spikelets was emasculated and dusted with pollen of H1175. Only 29 spikelets set seed and 12 seedlings were recovered after germination in sterilized soil. However, three plants died in the field soon after transplanting, so the effective crossability was very low: 0.41%. In these crosses, the hybrid nature of the progeny was assessed through molecular markers because the morphological characteristics of the parents would not allow identifying the hybrid origin without any doubt. Genomic DNA from both parents was screened in order to identify specific bands from the

**Table 2.** Morphological phenotypic characteristics of 4x *Paspalum simplex* (11B1a), 4x *P. malacophyllum* (TK-2449), and 20 individuals of their F<sub>1</sub> hybrids

Character	<i>P. simplex</i> 11B1a (female parent)	<i>P. malacophyllum</i> TK-2449 (pollen donor)	20 F <sub>1</sub> hybrids
Length of leaf blades (cm) <sup>a</sup>	<b>20.4</b> (14–23)	<b>39.6</b> (28–46)	<b>33.6</b> (25–42)
Maximum width of the leaf blades (cm) <sup>a</sup>	<b>0.9</b> (0.6–1.3)	<b>2.7</b> (2–3)	<b>1.6</b> (1.3–2.5)
Distance from the place of maximum leaf blade width up to the base of the blade (cm) <sup>a</sup>	<b>6.0</b> (4.5–9)	<b>19.4</b> (15–24)	<b>12.7</b> (8–20)
Width of the leaf blade at one cm above its basal end (cm) <sup>a</sup>	<b>0.6</b> (0.4–0.8)	<b>0.2</b> (0.15–0.25)	<b>0.48</b> (0.2–0.9)
Number of racemes per inflorescence	<b>13.5</b> (11–16)	<b>35.6</b> (28–58)	<b>32.5</b> (19–40)
Length of the central axis of the inflorescence (cm)	<b>13.2</b> (9–17)	<b>26.7</b> (19–45)	<b>23.5</b> (14–29)
Length of the basal raceme (cm)	<b>4.6</b> (3.5–6.2)	<b>10.4</b> (7–14)	<b>8.0</b> (6–11)
Length of the uppermost raceme of the inflorescence (cm)	<b>2.6</b> (1.4–4.2)	<b>2.2</b> (1–3.7)	<b>2.7</b> (1.1–4.6)

Mean and range (between parentheses) of 10 records for each parent and five records for each hybrid.

<sup>a</sup>Measurements implicate the third leaf below the inflorescence.

male parent *P. asterii* H1175. Forty arbitrary decamer oligonucleotides from two RAPD Primer Sets of the University of British Columbia were evaluated. A total of eight primers was selected because they generate several bands specific to the male parent. As a total, 65 bands were observed to be specific to *P. asterii* H1175. The nine progenies recovered from the *P. simplex* C1-2 × *asterii* H1175 crosses produced specific bands derived from the pollen donor. Most bands specific to the male parent were shared by the entire offspring, with a minimum of 37 to a maximum of 47, indicating that each descendant was a hybrid.

#### ***Paspalum simplex* Q4109 × *malacophyllum* V14855**

The crosses between these diploid accessions involved a single inflorescence of a self-incompatible plant of *P. simplex* as female parent. Two hundred and thirty-seven grains were formed from 579 pollinated spikelets. Only a sample of 50 filled spikelets was sown and 37 seedlings were recovered. In view of the self-incompatible nature of the female parent, all seedlings were assumed to be of hybrid origin. The morphological characteristics of the progeny concerning leaf shape and size, being intermediate between parents, left little doubt about the hybrid origin of these offspring. The leaf width in *P. simplex* ranged from 0.5 to 1 cm, and in diploid *P. malacophyllum* from 2 to 2.7 cm, while all offspring had leaves much wider than the female parent, varying between 1.2 and 2 cm. The degree of crossability was not established due to the fact that only one sample of seed was germinated. Out of the sub-sample grown, it could be estimated to be around 30% considering the percentage of spikelets that set seed and the proportion of seed that produced hybrid plants.

#### **Chromosome numbers of the hybrids**

Ploidy level of hybrids between 2x *P. simplex* and 2x *P. malacophyllum* was expected to be diploid. Therefore, a small sample of two individual plants was checked to substantiate this assumption, and both hybrids showed  $2n = 20$ .

Chromosome counting was performed for a large fraction of the total number of plants classified as hybrids in those crosses involving the induced tetraploid materials of *P. simplex* as female parent. The counts were considered a necessary requisite because plant 11B1a is a mixoploid with some diploid tillers but with mainly tetraploid sectors, and because of the meiotic irregularities observed in the male parents. Due to the high number of *P. simplex* × *procurrens* hybrids, only a 10-plant sample was chosen randomly for chromosome counts in this cross.

The 10 *P. simplex* 11B1a × *procurrens* Q4094 sample hybrids, and all the 22 *P. simplex* 11B1a × *malacophyllum*

TK2449 hybrids had  $2n = 4x = 40$  chromosomes, as expected if the emasculated inflorescences employed for crosses belonged to the tetraploid sector of the mixoploid (2x–4x) 11B1a plant.

The nine hybrids from *P. simplex* 11B1a × *malacophyllum* V5095 were triploid,  $2n = 3x = 30$ , a result that could be expected only if the inflorescence used for this cross came from a rare diploid tiller of the mixoploid 11B1a plant. Since this plant had been obtained by treating seed of diploid Q4109 accession of *P. simplex* with colchicine, and because the triploid hybrids arose from a non-duplicated sector of the 11B1a plant, we judged the origin of these nine hybrids to be a cross between diploid *P. simplex* Q4109 and tetraploid *P. malacophyllum* V5095, to be identified subsequently as *P. simplex* Q4109 × *malacophyllum* V5095 triploid hybrids.

Chromosome number was established for the nine *P. simplex* × *asterii* hybrids that survived. Six hybrids had  $2n = 40$  chromosome as expected in a  $4x \times 4x$  cross. Two hybrids had  $2n = 38$  and one had  $2n = 39$  chromosomes. These aneuploids probably arose from gametes lacking one or two chromosome due to meiotic irregularities in the parental plants.

#### **Meiotic chromosome behavior**

Meiotic chromosome configurations were investigated in parental species and their hybrids, except for diploid *P. simplex* whose regular meiotic behavior has been previously reported (Espinoza and Quarin, 1997). Results are summarized in Table 3. The diploid accession of *P. malacophyllum* showed a fairly regular meiotic behavior with 10 chromosome bivalents in the majority of the observed PMC. Chromosomes were symmetrically distributed during anaphase of the first meiotic division.

As expected, most chromosomes of the induced autotetraploid *P. simplex* associated at meiosis forming mainly quadrivalents and bivalents. Despite the fact that the tetraploid plant 11B1a arose directly from colchicine treatment of a diploid seed (generation C<sub>0</sub>), nearly 40% of the chromosomes failed to form quadrivalent associations and paired as bivalents.

Chromosome pairing in all the natural tetraploid material, *P. procurrens* Q4094, *P. malacophyllum* TK2449 and V5095, *P. asterii* H1175, showed a high proportion of quadrivalent associations at diakinesis and metaphase I. Examples of meiotic chromosome pairing are illustrated in Fig. 1a (*P. procurrens* Q4094) and Fig. 1b (*P. malacophyllum* V5095).

Two individual hybrid plants of 2x *P. simplex* Q4109 × 2x *malacophyllum* V14855 had fairly regular meiotic behavior with mainly 10 chromosome bivalent at diakinesis and metaphase of the first division (Table 3), and showed a subsequent normal homologous

**Table 3.** Meiotic chromosome configurations observed at diakinesis and metaphase I in four *Paspalum* species and their interspecific hybrids

Species	$2n$	Scored plants	No. PMC	Mean chromosome associations per PMC and range per PMC (between parentheses)								
				I	II	III	IV	V	VI	VIII		
<i>P. simplex</i> Q4109 <sup>a</sup>	20	Several	120	–	<b>10.0</b>	–	–	–	–	–	–	–
<i>P. malacophyllum</i> V14855	20	1	214	<b>0.019</b> (0–2)	<b>9.99</b> (9–10)	–	–	–	–	–	–	–
<i>P. simplex</i> 11B1a Colchicine-induced 4x	40	1	68	<b>0.55</b> (0–6)	<b>7.66</b> (2–16)	<b>0.11</b> (0–2)	<b>5.94</b> (2–9)	–	–	–	–	–
<i>P. malacophyllum</i> V5095	40	1	36	<b>0.08</b> (0–2)	<b>8.52</b> (2–14)	<b>0.02</b> (0–1)	<b>4.8</b> (2–9)	–	–	–	–	<b>0.44</b> (0–1)
<i>P. malacophyllum</i> TK2449	40	3	40	<b>0.075</b> (0–2)	<b>11.7</b> (5–18)	<b>0.025</b> (0–1)	<b>4.0</b> (1–7)	–	–	<b>0.075</b> (0–1)	–	–
<i>P. procurrans</i> Q4094	40	3	53	<b>0.06</b> (0–2)	<b>10.0</b> (4–16)	–	<b>4.96</b> (2–8)	–	–	–	–	–
<i>P. usterii</i> H1175	40	1	55	<b>2.04</b> (0–8)	<b>8.88</b> (3–16)	<b>1.4</b> (0–4)	<b>4.05</b> (1–8)	<b>0.07</b> (0–1)	<b>0.03</b> (0–1)	<b>0.02</b> (0–1)	<b>0.02</b> (0–1)	–
<b>Hybrids</b>												
<i>P. simplex</i> Q4109 × <i>malacophyllum</i> V14855	20	2	249	<b>0.084</b> (0–4)	<b>9.8</b> (8–10)	–	<b>0.08</b> (0–1)	–	–	–	–	–
<i>P. simplex</i> Q4109 × <i>malacophyllum</i> V5095	30	4	162	<b>2.14</b> (0–7)	<b>2.14</b> (0–7)	<b>7.86</b> (3–10)	–	–	–	–	–	–
<i>P. simplex</i> 11B1a × <i>malacophyllum</i> TK2449	40	3	113	<b>1.34</b> (0–6)	<b>12.78</b> (4–18)	<b>0.32</b> (0–2)	<b>3.03</b> (1–8)	–	–	–	–	–
<i>P. simplex</i> 11B1a × <i>procurrans</i> Q4094	40	3	73	<b>1.75</b> (0–6)	<b>13.62</b> (2–20)	<b>0.27</b> (0–3)	<b>2.55</b> (0–7)	–	–	–	–	–
<i>P. simplex</i> C1-2 <sup>b</sup> × <i>usterii</i> H1175	40	2	104	<b>0.97</b> (0–6)	<b>9.02</b> (0–16)	<b>0.7</b> (0–3)	<b>4.1</b> (2–10)	<b>0.12</b> (0–1)	<b>0.13</b> (0–1)	<b>0.04</b> (0–1)	<b>0.04</b> (0–1)	–

<sup>a</sup>From Espinoza and Quarín (1997). Several plants from the original Q4109 accession were analyzed. Seeds of diploid accession Q4109 were originally used by Cáceres et al. (1999) to induce sexual tetraploid plant 11B1a.

<sup>b</sup>C1-2 is a sexual 4x plant derived from seed of self-pollinated colchicine-induced 11B1a.

chromosome distribution at anaphase. This regular meiotic chromosome pairing in the interspecific hybrids indicated a close genomic homology between the basic chromosome sets of *P. simplex* and *P. malacophyllum*. Occasionally, a quadrivalent chromosome association was observed during diakinesis and metaphase I. This multivalent association was not expected since the hybrids were diploid. It is assumed that this quadrivalent, observed in some PMC of each of the two hybrids analyzed, was an indication that diploid *P. simplex* and diploid *P. malacophyllum* differed at least in one chromosome translocation.

Meiotic chromosome behavior was examined in four triploid hybrids of *P. simplex* Q4109 × *malacophyllum* V5095. A total of 162 PMCs was analyzed at diakinesis and metaphase I. Most chromosomes formed trivalent associations (Table 3 and Fig. 1c). A mean of 7.86 trivalents (range 3–10) with a maximum of 10 per PMC indicated a close homology between the genome of diploid *P. simplex* and the two genomes afforded by tetraploid *P. malacophyllum* V5095. Furthermore, when hybridization involved the two species at tetraploid level (induced 4x *P. simplex* 11B1a × *malacophyllum* TK2449) the hybrids showed a high proportion of their chromosomes associated to form bivalents and quadrivalents at meiosis. The mean quadrivalent chromosome associations averaged 3.08 per PMC with some cells showing up to eight quadrivalents (Table 3).

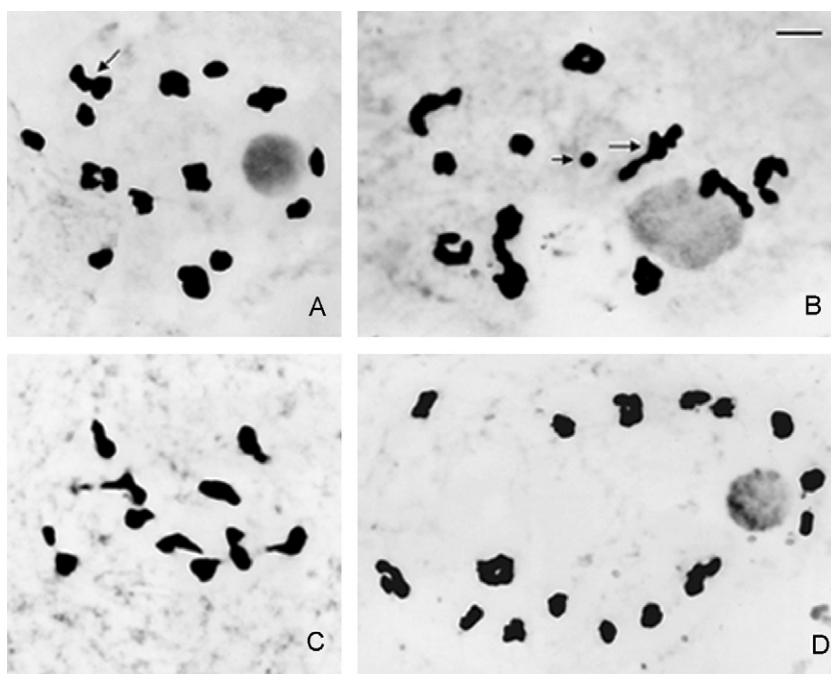
Three interspecific hybrids among 4x *P. simplex* 11B1a and 4x *P. procurrans* Q4094 were cytologically analyzed at diakinesis and metaphase of the first meiotic division. The 40 chromosomes associated mainly as bivalents and quadrivalents, with means = 13.62/PMC and 2.55/PMC, respectively (Fig. 1d). Up to seven quadrivalents were observed per PMC (Table 3).

Meiosis was analyzed in two interspecific tetraploid hybrids of *P. simplex* C1-2 × *usterii* H1175; both showed most associated chromosomes forming bivalents (mean = 9.02/PMC) and quadrivalents (mean = 4.1/PMC) with some univalents and trivalents, and few other multivalent associations (Table 3). These multivalent chromosome associations, higher than quadrivalents, could be attributed to chromosome changes, most likely chromosome translocations, which had occurred in one of the parental species.

## Fertility

The selected diploid plants of *P. simplex* and *P. malacophyllum* failed to set seed when inflorescences were isolated from pollen of other genotypes, yet over 70% of the spikelets formed grains in 2x *P. simplex* when inflorescences were allowed to receive pollen





**Fig. 1.** Meiotic chromosome associations in two *Paspalum* species and two interspecific hybrids: (A) tetraploid *P. procurrens* Q4094, diakinesis with 10 bivalents + 5 quadrivalents [observe two bivalents close together (arrow)]. (B) Tetraploid *P. malacophyllum* V5095, diakinesis with 1 univalent (short arrow) + 2 bivalents + 1 trivalent (long arrow) + 6 quadrivalents + 1 octovalent. (C) Triploid interspecific hybrid, *P. simplex* Q4109 × *malacophyllum* V5095, metaphase I with 10 trivalents. (D) Tetraploid interspecific hybrid, *P. simplex* 11B1a × *procurrens* Q4094, diakinesis with 12 bivalents + 4 quadrivalents.

freely from other plants of the population (Table 4). These results confirmed that  $2x$  races of *P. simplex* are sexual, self-sterile but cross-fertile, as reported earlier (Espinoza and Quarin, 1997). The incapacity of diploid *P. malacophyllum* for seed setting under self-pollination condition is an indication of self-sterility. Since we had only two genotypes of this diploid strain, and because they overlapped their flowering periods during a very short time, the low seed set under open pollination (2%) might not reveal the real outbreeding potential of these plants. These results, though incomplete, suggest that the diploid cytotype of *P. malacophyllum* is sexual and self-sterile due to a self-incompatibility genetic system, as is the rule for most diploid races of those *Paspalum* species involving sexual diploid and apomictic polyploid races (Quarin, 1992).

Our results indicate that the induction of autotetraploidy from the sexual self-sterile diploid *P. simplex* reported by Cáceres et al. (1999) had little effect on the self-incompatibility system. Less than 2% of the florets set seed when inflorescences of induced tetraploid plant 11B1a were selfed, while almost 45% produced grain under open pollination (Table 4).

The three natural tetraploid species: *P. procurrens*, *P. malacophyllum* (accessions TK2449 and V5095), and *P. usterii* set seed under open pollination and also when selfed (Table 4). However, the degree of seed setting was

very variable as characteristic of most apomictic tetraploid species of *Paspalum*.

Fertility was very erratic among the different interspecific hybrids obtained in this work. Some hybrids were sterile while others showed a wide array of fertility, from 0.02% in open-pollinated  $F_1$  #1 of *P. simplex* C1-2 × *usterii* H1175 to 36.6 in backcrossed  $F_1$  45 of *P. simplex* 11B1a × *procurrens* Q4094 (Table 4). The low degree of fertility in  $F_1$  hybrids of diploid *P. simplex* × diploid *P. malacophyllum* was unexpected since their meiotic chromosome behavior was fairly normal with mainly bivalent pairing. Seed production among the triploid hybrids *P. simplex* Q4109 × *malacophyllum* V5095 varied from sterility (0%) to 9.2%. The sterility or low fertility was attributable to meiotic irregularities caused by triploidy. The individual hybrid plant  $F_1$  #3, which had the highest fertility level (9.2%), was apomictic and most of its  $F_2$  descendants originated by  $2n+n$  fertilization (data not shown). The fertility of tetraploid interspecific hybrids *P. simplex* 11B1a × *procurrens* Q4094 and *P. simplex* 11B1a × *malacophyllum* TK2449 in backcrossing experiments, though varying among individuals, showed fairly high levels corroborating the close phylogenetic relationship of the three species, and the feasibility of gene transferring among them when sexuality is available at the tetraploid level. The degree of fertility in tetraploid

**Table 4.** Seed fertility of four *Paspalum* species and their interspecific hybrids

Parental species and hybrids	Percentage of spikelets that formed grain after		
	Self-pollination	Open-pollination	Backcrossed to male parent
<i>P. simplex</i> Q4109 (plant # 59) sexual 2x	0.0	70.2	–
<i>P. malacophyllum</i> V14855 sexual 2x	0.0	2.1 <sup>a</sup>	–
<i>P. simplex</i> 11B1a sexual induced 4x	1.9	44.9	–
<i>P. simplex</i> C1-2 sexual 4x	0.05	39.8	–
<i>P. procurrens</i> Q4094 apomictic natural 4x	16.7	42.8	–
<i>P. malacophyllum</i> TK2449 apomictic natural 4x	22.4	34.6	–
<i>P. malacophyllum</i> V5095 apomictic natural 4x	7.2	13.3	–
<i>P. usterii</i> H1175 apomictic natural 4x	5.88	5.8	–
<i>P. simplex</i> Q4109 (2x) × <i>malacophyllum</i> V14855 (2x)			
F <sub>1</sub> 02	0.5	1.2	–
F <sub>1</sub> 22	0.0	0.2	–
<i>P. simplex</i> Q4109 (2x) × <i>malacophyllum</i> V5095 (4x)			
F <sub>1</sub> 03	0.0	2.7	9.2
F <sub>1</sub> 06	0.0	0.0	0.0
F <sub>1</sub> 07	0.0	0.05	0.5
F <sub>1</sub> 08	0.0	0.3	3.0
F <sub>1</sub> 09	0.0	0.0	0.0
<i>P. simplex</i> 11B1a (4x) × <i>malacophyllum</i> TK2449 (4x)			
F <sub>1</sub> 12	1.5	3.3	20.2
F <sub>1</sub> 16	0.4	3.7	17.5
F <sub>1</sub> 17	1.9	3.6	3.1
<i>P. simplex</i> 11B1a (4x) × <i>procurrens</i> Q4094 (4x)			
F <sub>1</sub> 22	4.9	4.3	8.2
F <sub>1</sub> 45	0.6	13.4	36.3
F <sub>1</sub> 47	1.7	26.6	33.2
F <sub>1</sub> 65	2.3	6.5	22.8
F <sub>1</sub> 66	0.1	4.6	4.1
<i>P. simplex</i> C1-2 (4x) × <i>usterii</i> H1175 (4x)			
F <sub>1</sub> 01	0.0	0.02	–
F <sub>1</sub> 02	0.12	0.36	–
F <sub>1</sub> 08	0.32	0.24	–
F <sub>1</sub> 11	0.0	0.9	–

<sup>a</sup>Only one different genotype, with limited flowering, was placed in the vicinity.

interspecific hybrids *P. simplex* × *usterii* (Table 4) was below 1%, indicating a less significant phylogenetic relationship between *P. simplex* and *P. usterii* than was observed among *P. simplex*, *P. procurrens* and *P. malacophyllum*.

## Discussion

Cytological analysis of interspecific hybrids indicated that 2x and 4x cytotypes of *P. simplex*, *P. procurrens*, and *P. malacophyllum*, and a 4x cytotype of *P. usterii* have basically homologous genomes. The tetraploid races of all these species originated by autopolyploidy. Facultative apomixis of the aposporous type is reported

for the first time in tetraploid strains of *P. procurrens* and *P. usterii*. It is also confirmed for tetraploid *P. malacophyllum*, a species whose diploid representative is here reported to reproduce sexually with some potential for apomixis.

As expected, the diploid accession V14855 of *P. malacophyllum* has regular meiotic behavior and sexual reproduction, and is probably self-incompatible since this genotype failed to produce grain when grown in an isolated environment. However, it was not sterile because some seed was recovered at the end of its flowering phase when another genotype of the same species and ploidy level started flowering in the same greenhouse. This sexually reproducing diploid has some potential for apomictic reproduction because an aposporous embryo sac was sporadically observed beside the typical meiotic sac and

inside of the same ovule. Additional research is needed to determine whether further parthenogenesis occurs in this occasional aposporous sac to produce a descendant via apomixis, or whether fertilization of the unreduced egg cell with pollen of  $4x$  plants would raise the ploidy level in the progeny. In this condition, ploidy may also increase by two steps through a triploid bridge. If this occurs, the relationship between  $2x$  and  $4x$  levels might be a dynamic system of one-way traffic carrying genetic variation from diploid outbreeders to apomictic tetraploids. This would be an effective dynamic system in a frame of autopolyploidy and apomixis, but different from the two-way traffic increasing and decreasing genome dosage in other plant species as suggested by Bennett (2004).

A high proportion of chromosomes associated forming quadrivalents at meiosis in tetraploid *P. procurrens*, *P. usterii* and both accessions of tetraploid *P. malacophyllum*. The number of quadrivalents observed in these two accessions was somewhat lower than reported by Bashaw et al. (1970) and considerably higher than reported by Burson and Hussey (1998). Nonetheless, these authors classified *P. malacophyllum* as autotetraploid. Our results support this classification for this species and also for *P. procurrens* and *P. usterii*. In the genus *Paspalum* many apomictic tetraploid species have sexual diploid counterparts; it has been suggested that this should be a typical feature for most apomictic polyploid species of the genus (Quarin, 1992). Our research provides information supporting that this scheme, with sexual diploid and apomictic autotetraploid representatives, is the most common system among species of *Paspalum*: the facultative apomictic tetraploid accession of *P. procurrens* is added to the sexual diploid representative reported previously (Quarin, 1993), and accession V14855 of *P. malacophyllum* indicates that sexual diploid races exist for this species. However, the sexual diploid representatives seem to be much less frequent in the wild than are the tetraploid races of *P. malacophyllum*.

We determined the mode of reproduction when those accessions were used as male parents in the interspecific crosses. Any given material was assumed to reproduce sexually when the embryo sac developed from a functional megaspore which originated by meiosis. Accessions in which the embryo sacs may develop from cytologically unreduced nucellar cells (apospory) were assumed to reproduce by means of apomixis. If apospory is the rule and meiotic embryo sacs fail to develop, the accession would be classified as obligate apomictic. In consequence, the diploid material of *P. malacophyllum* is classified as a sexually reproducing race with some potential for apomixis, while both tetraploid accessions of this species are facultative apomicts. Accession TK2449 showed the highest degree of apomictic reproduction since only 3% of its mature ovules had a single embryo sac of the meiotic type (with

proliferated antipodal cells), while in accession V5095, 17% of the ovules showed a single meiotic sac. Variations in the degree of facultative apomixis had been observed in several tetraploid accessions of *P. malacophyllum* analyzed by Burson and Hussey (1998) through embryological studies. However, the degree of sexuality in these apomictic accessions needs further confirmation. Aposporous embryo sacs bearing antipodal cells (*Hieracium*-type) were observed in tetraploid strains of *P. simplex*, a species of the same subgenus. If *Hieracium*-type of apospory occurs in *P. malacophyllum*, the degree of sexual reproduction may be largely overestimated. Actually, Bashaw et al. (1970), based on embryological studies, reported that a 40-chromosome accession of this species was sexual. The apomictic mode of reproduction reported by Burson and Hussey (1998) in 14 accessions, and in two additional accessions in the present study, leaves little doubt about the facultative apomictic reproduction system in tetraploid *P. malacophyllum*, but progeny tests would elucidate what is the real degree of sexuality. Our embryological studies indicate that the tetraploid strains of *P. procurrens* reproduce by facultative apomixis of the aposporous type, while diploid races of the same species were reported to be sexual outbreeders due to the operation of a self-incompatibility system (Quarin, 1993). *Paspalum usterii* is a tetraploid species that reproduces by facultative apomixis. The embryo sac types observed in mature ovules indicate a higher degree of sexuality than that observed for both the tetraploid accession of *P. malacophyllum* and also for tetraploid *P. procurrens*. Nevertheless, the low number of antipodal cells observed in the gametophytes that were classified as meiotic embryo sacs emphasizes some doubt in relation to the origin of these sacs. Thus, further investigation and other experimental approaches would be required to establish the actual degree of sexual reproduction of this facultative apomictic species.

Because sexual diploid strains usually exist for apomictic tetraploid *Paspalum* species, it seems predictable to find sexual diploid representatives for *P. usterii* in extensive collections of the species.

The meiotic chromosome associations observed in the parental species and in their interspecific hybrids suggest on the one hand the autopolyploid origin of tetraploid races and, on the other hand, the close genomic relationship among species of the subgenus *Anachyris*. Hybrids between *P. simplex* and *P. malacophyllum* at the diploid level showed fairly regular meiotic chromosome pairing as evidence of their homology. When the interspecific hybrids were obtained at the tetraploid level, most chromosomes associated forming bivalent and quadrivalent associations as is the rule in the parental species. Moreover, the triploid interspecific hybrids ( $2x$  *P. simplex*  $\times$   $4x$  *malacophyllum*,  $2n = 3x = 30$ ) showed

up to ten trivalent chromosome associations in some cells. The whole interpretation of meiotic chromosome pairing of interspecific hybrids between *P. simplex* and *P. malacophyllum* at different ploidy levels supports a common origin and almost complete homology among their genomes. A similar degree of homology was previously found among diploid races of *P. simplex* and *P. procurrens* (Espinoza and Quarin, 1998). The meiotic chromosome associations that we observed in tetraploid interspecific hybrids of *P. simplex* × *procurrens* confirm previous reports since most chromosomes associated as bivalents and quadrivalents with a maximum of seven quadrivalents per cell. However, the tetraploid interspecific hybrids *P. simplex* × *procurrens* showed a lower proportion of quadrivalents per cell (mean = 2.55) than those observed in their parents (means of 5.94 for *P. simplex* and 4.96 for *P. procurrens*). This would indicate that some changes have occurred in the speciation processes that affected their genomes, though basically they preserve their homology. The meiotic chromosome behavior of *P. simplex* × *usterii* tetraploid hybrids showed a high proportion of chromosomes associated as bivalents (mean = 9.02/cell) and quadrivalents (mean = 4.1/cell), with a maximum of 10 quadrivalents/cell. In view of the experimental autopoloid origin of *P. simplex*, and the high proportion of quadrivalent associations in the hybrids, tetraploid *P. usterii* is to be classified as an autotetraploid with a basic genome homologous to the genome of *P. simplex*. The occasional chromosome associations higher than quadrivalent observed in *P. simplex* × *usterii* tetraploid hybrids suggest some chromosome rearrangements, probably translocations.

The present results, in addition to previous studies of  $2x$  *P. simplex* ×  $2x$  *procurrens* hybrids, suggest that the whole subgenus *Anachyris* of *Paspalum* is basically an agamic complex for which six species are actually recognized: *P. simplex* (with sexual  $2x$ , sexual and apomictic  $3x$ , and apomictic  $4x$  and  $6x$  representatives), *P. malacophyllum* (sexual  $2x$  and apomictic  $4x$ ), *P. procurrens* (sexual  $2x$  and apomictic  $4x$ ), *P. usterii* (only apomictic  $4x$  races known), *P. volcanensis* ( $4x$ ), and *P. costellatum* for which no information on ploidy level or mode of reproduction is available. Our experimental research results indicate that the same basic genome is shared by at least four of the six species of the subgenus: *P. simplex*, *P. malacophyllum*, *P. procurrens*, and *P. usterii*. In these species the polyploid representatives originated by autopoloidy and all reproduce by means of facultative apomixis with the exception of a rare triploid plant of *P. simplex* which reproduces sexually, though another  $3x$  plant of the same species is apomictic (Urbani et al., 2002). The sexual tetraploid plants of *P. simplex* used in this work were obtained experimentally by colchicine treatment of diploid plants. Sexual tetraploid individuals have never

been found in natural populations of any of these species. The results suggest that speciation took place at the diploid level, then polyploidization, apomixis, and eventually geographic barriers prevented hybridization and introgression. For example, experimental work demonstrated that *P. simplex* and *P. procurrens*, at the diploid level, hybridize and backcross very easily. However, the diploid races of these species retain geographical separation, although both belong to the same general phytogeographic Chaco region in northern Argentina (Espinoza and Quarin, 1998). The unique diploid representative of *P. malacophyllum*, as studied so far, was collected in the state of Santa Catarina, Brazil. Although our crosses between diploid races of *P. simplex* and *P. malacophyllum* produced barely fertile hybrids, introgression in the wild has been primarily prevented by a wide geographic barrier. Where these species distribution overlap as in areas of northern Paraguay or southwestern Brazil, introgression should be hardly accomplished due to the polyploid apomictic nature of these species. Interestingly enough, the general morphological characteristics of the diploid accession of *P. malacophyllum* resemble the unusual accession V5095 of *P. malacophyllum* which is tetraploid and apomictic and was originally collected in the state of Goiás, Brazil. The uncommon general morphology, the decumbent growth habit of this material, and the fact of their existence at both diploid and tetraploid levels, requires a special consideration of their taxonomic status. They clearly differ from the original description and illustration reported by Trinius (1831) when describing *P. malacophyllum*. Additional botanical collections and information regarding distribution and population characteristics of material with these unusual morphological attributes would help in the development of an improved taxonomic classification.

## Acknowledgements

This study was financed by grants from Agencia Nacional de Promoción Científica y Técnica (AN-PCYT), the Secretaría General de Ciencia y Técnica, Universidad Nacional del Nordeste (UNNE), Argentina, and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasil. We thank the apomixis research staff of the Institute of Plant Genetics-CNR, Perugia, Italy, for their cooperation and for providing sexual plant material of tetraploid *P. simplex*. Martínez and Quarin are members of the research staff, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Hojsgaard was funded by a fellowship from CONICET; Schegg was an undergraduate student in the Universidad Nacional del Nordeste, Corrientes, Argentina, and Valls holds a Research Productivity Grant from CNPq, Brazil. We

thank Professor Michael D. Hayward, Aberystwyth, Wales and Henry A. Fribourg, Professor Emeritus of Crop Ecology, University of Tennessee, USA for their critical review and their suggestions related to English grammar.

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