Diversity in Diploid, Tetraploid, and Mixed Diploid–Tetraploid Populations of *Paspalum simplex*

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ABSTRACT

The tetraploid cytotype of *Paspalum simplex* Morong has a wide geographical distribution and reproduces by apomixis while the diploid grows in a restricted area in northern Argentina and behaves as cross-pollinated. The objective was to evaluate the genetic diversity among and within natural diploid and tetraploid populations of *P. simplex* growing in sympatry and allopatry by studying the phenotypic variability and DNA polymorphisms. Twenty-three phenotypic traits and 10 inter-simple sequence repeat (ISSR) molecular markers were used for evaluating the variability present in six populations of *P. simplex*. Two populations were diploid and four were tetraploid. One of the tetraploid populations was sympatric to a diploid population while the rest of the populations were allopatric. A rich diversity was observed for specific traits, especially for seasonal biomass yield, presence of a terminal raceme in the inflorescence, and leaf pubescence. The 2*x* and 4*x* populations growing in proximity were closely related. Diversity was higher within diploid populations when compared with the evaluated tetraploid populations. Among the tetraploid populations, the one sympatric with a diploid population exhibited the greatest diversity. Gene flow occurs between 2*x* sexual and 4*x* apomictic populations of *P. simplex*. Diploid populations seem to be the main source of the diversity observed among tetraploid populations.

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; ISSR, inter-simple sequence repeat; TAE, Tris-acetate-ethylenediaminetetraacetic acid; UV, ultraviolet.

Asexual reproduction is usually associated with the formation of genetically uniform populations. Apomictic species are expected to follow this pattern because the offspring are identical to the mother plant. However, a rich diversity is commonly found in apomictic species, such as apomictic *Ranunculus carpaticola* Soó (Paun et al., 2006), *Paspalum notatum* Flüggé (bahiagrass) (Espinoza et al., 2006; Cidade et al., 2008; Reyno et al., 2012), and *Megathyrsus maximus* (Jacq.) B. K. Simon & S. W. L. Jacobs (syn. *Panicum maximum* Jacq.) (guineagrass) (Barbosa de Sousa et al., 2011). Most of this diversity is expected to be present among rather than within populations (Stebbins, 1950; Hörandl and Paun, 2007). Moreover, apomictic populations are usually dominated by one or a few genotypes, which are well adapted to a particular habitat (Richards, 1990). Diversity in apomictic species is considered the result of cross-pollination with sexual relatives, facultative sexuality within apomicts, mutations, and divergence among the sexual ancestors (Hörandl and Paun, 2007). However, the importance of each of these factors for the generations of variability remains unknown for most apomictic species.

Paspalum L. is a grass genus that represents an interesting model for studying the sources of genetic variation in apomictic species. This genus has 400 species, which are characterized by a

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complex genetic system with variation in ploidy levels and reproductive modes (Quarin, 1992). Approximately 60% of the species that have been characterized are apomictic, and these are all polyploid. In addition, species with different ploidy levels are commonly found in the genus. Examples of these species are *P. notatum* with naturally occurring 2*x*, 3*x*, 4*x*, and 5*x* cytotypes (Gates et al., 2004) and *P. simplex* with 2*x*, 3*x*, 4*x*, and 6*x* races (Urbani et al., 2002). In these multiploid species apomixis is linked to polyploidy, and sexuality only occurs in diploid plants, which are usually cross-pollinated. *Paspalum notatum* and *P. simplex* are important components of rangelands in South America, and these species are used as representatives of the genus for genetic and evolutionary studies.

Several studies have shown that cross-pollination occurs between apomictic and sexual populations of the same or related species (Hörandl and Paun, 2007). This phenomenon results in 4*x* apomictic populations sympatric to 2*x* sexual ones being more diverse than apomictic populations growing in isolation. One interesting example is the genus *Taraxacum* F. H. Wigg. section *Ruderalia*, which contains sexual diploid and apomictic triploid plants, and novel genotypic variation is the result of gene flow between these two groups (Menken et al., 1995). Gene flow from 2*x* sexual to 4*x* apomictic populations was proposed to explain the different degrees of genetic and genotypic variation observed in 4*x* populations of *P. notatum* growing in sympatry or allopatry with diploids (Daurelio et al., 2004). This species has a vast geographic distribution, and most of the area is occupied by tetraploid biotypes. Diploid populations are restricted to a small area in northeastern Argentina. High values of genotypic and genetic variability were observed within the 4*x* population growing in close proximity with diploid ones while almost all individuals were genetically identical in the allopatric 4*x* population, and the few distinguishable ones showed a genetic constitution similar to the main clone.

Paspalum simplex is an interesting species to study because it is also a multiploid species, and sympatric, mixed diploid and polyploid, and pure diploid and tetraploid populations have been reported (Urbani et al., 2002). The species grows naturally in northern Argentina, Paraguay, northeastern Uruguay, and eastern Bolivia. Diploid populations are only found in north central Argentina while the rest of the area is inhabited mainly by tetraploid biotypes. Although *P. simplex* and *P. notatum* are not closely related species, they share a genetic system where diploid plants are sexual and outbreeders while polyploids are apomictic. Therefore, if these two species share a pattern of genotypic diversity within and among populations, general conclusions about the genus could be drawn. In particular, it is necessary to better understand how novel apomictic genotypes are generated in *Paspalum*.

Paspalum simplex and *P. notatum* are also appreciated by the local ranchers because the rangelands where these species grow are considered highly productive. Several species of *Paspalum* are currently being genetically improved in the United States, Brazil, and Argentina with the aim of generating new forage cultivars for subtropical regions. Information related to the diversity present in natural populations of *Paspalum* species is also needed to enhance these breeding programs.

The objective of this research was to evaluate the diversity contained among and within natural sexual diploid and apomictic tetraploid populations of *P. simplex* growing in sympatry and allopatry. For this purpose pure, 2*x* or 4*x*, and mixed 2*x*–4*x* populations were characterized based on their morphology, phenology, biomass yield, and DNA sequence.

MATERIALS AND METHODS

Plant Material

The germplasm for this research was collected from five different locations (Fig. 1) during summer 1998 and 1999. A representative sample of seed and small cuttings of rhizomes from each population was collected in the Argentina's provinces of

Figure 1. Map of Argentina indicating the location and ploidy level of the six studied populations of *Paspalum simplex*.

Chaco (27°35′ S, 60°44′ W), Corrientes (29°10′ S, 58°04′ W), Formosa (25°43′ S, 59°05′ W), Santa Fe (29°08′ S, 59°39′ W), and Santiago del Estero (27°08′ S, 61°50′ W); each collection covered approximately 2000 m^2. Cuttings were taken systematically every 10 m within each one of the sampled areas. Two of these populations were previously characterized as diploid ($2n = 2x = 20$) while the other four were characterized as tetraploid ($2n = 4x = 40$) (Urbani et al., 2002). At one of the collection sites, diploid and tetraploid plants were found, so this tetraploid population was described as sympatric to the diploid one (Fig. 1). The rest of the tetraploid populations were isolated from diploid populations and were identified as allopatric.

Morphological and Agronomic Characterization

Twenty individuals from each tetraploid population and 10 for each diploid population were used for the morphological characterization. Rhizomes from each plant were planted in pots with sterile soil in a greenhouse during winter 1999. Each plant was vegetatively propagated in 12 clones and transplanted into the field located in Corrientes, Argentina (27°28′45″ S, 58°47′06″ W), at the beginning of the spring 1999. The soil in that location was classified as Udipsamment alfico. A randomized complete block design with four replications was used for this study in which three plants were the experimental unit. A total of 1200 plants were transplanted on 1-m centers. The soil was treated with 125 cm^3 m^{-2} metham sodium (32.7% $C_2H_4NNaS_2$) 60 d before transplanting. Plants were fertilized with 3 g of 15–15–15 (N–P–K) per plant 10 d after transplanting and were kept well irrigated during the experiment.

Once the individual plants were established in the field, data for 23 traits was taken from each plant. The lengths of the basal, mid, and upper racemes were measured in two inflorescences of each plant. Leaf length and width were measured in the leaf immediately below the flag leaf considering only the leaf blade. Tiller and inflorescence length and number of racemes per inflorescence were measured together with the previously described traits in January 2000.

In February 2000, plants were classified as erect, semierect, or prostrate with the aim of characterizing their growth habit. Plants were also classified based on the color of the stigma and anther using three categories: yellow, light purple, and purple. Another trait that was considered was the presence or absence of a terminal raceme. In March 2000, the presence of hairs in leaf blades and sheaths and anthocyanins in spikelets were evaluated as absent, scarce, medium, and abundant. During the second growth period, beginning, peak, and end of the flowering period were weekly evaluated.

Biomass yield was also measured four times during two growing seasons. Plants were cut twice during the establishment season on 21 Feb. and 20 Apr. 2000 and twice during the second season on 15 Dec. 2000 and 23 Feb. 2001. For this purpose, individual plants were cut 10 cm above the soil surface, and the material was dried at 60°C for 48 h and weighted.

Molecular Characterization

Seed from each population was stored in a refrigerator between 1999 and 2009. Approximately 200 seeds from each population were sown in trays with sterile soil in a greenhouse in August 2009. Fifteen seedlings from each of the six populations were used for the DNA extraction and their further molecular analysis. Total genomic DNA was isolated using the Chen and Ronald (1999) procedure with the following modifications: 50 mg of young leaf was macerated with the help of a plastic fuse drill and 2% w/v cetyltrimethylammonium bromide buffer (100 mM Tris-HCl pH 7.5, 50 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 700 mM NaCl, and 140 mM β-mercaptoetanol) prewarmed at 65°C.

The concentration and integrity of the DNA samples were determined by electrophoresis in 1% agarose gels in 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 5 mM NaOAc, and 0.77 mM EDTA) at 40 V for 2 h. A dilution series of a DNA standard of known concentrations were used to estimate the concentration of each DNA sample. Genomic DNA was visualized under ultraviolet (UV) light and photographed with GelDoc-It Imaging System (UVP, LLC) after staining with ethidium bromide (10 mg mL^{-1}). All samples were adjusted to 10 ng μL^{-1} for their use in polymerase chain reaction amplifications.

A total of 10 primers (PROMEGA) were used for inter-simple sequence repeat (ISSR) amplifications. The ISSR reaction was performed as described by Cidade et al. (2008). Polymerase chain reaction products were separated by electrophoresis in 2% agarose gels in 1x TAE buffer at 70 V for 3 h and stained with ethidium bromide (10 mg mL^{-1}). The profiles were visualized under UV light, photographed, and stored for further analysis with GelDoc-It Imaging System (UVP, LLC). Table 1 contains the information about the analyzed primers, number of amplified markers, number of polymorphic markers, polymorphism index, and polymorphism information content obtained with each primer.

Table 1. Inter-simple sequence repeat molecular characterization of six populations of *Paspalum simplex*: primers analyzed, number of amplified markers, number of polymorphic markers, polymorphism index, and polymorphism information content (PIC) obtained with each primer.

Primer repeat 5′→3′	Number of amplified markers	Number of polymorphic markers	Polymorphism index (%)	PIC (mean ± SE)
(TC)8-A	7	7	100	0.14 ± 0.05
(CA)8-T	14	13	92.8	0.08 ± 0.02
(GA)8-T	17	17	100	0.22 ± 0.03
(AC)8-G	18	18	100	0.20 ± 0.03
(GT)8-C	18	18	100	0.17 ± 0.03
(GA)8-C	17	17	100	0.20 ± 0.03
(AC)8-T	15	15	100	0.21 ± 0.03
(CT)8-G	15	15	100	0.21 ± 0.03
(AG)8-C	16	16	100	0.19 ± 0.03
(CA)8-G	3	2	66.6	0.02 ± 0.03

Ploidy Level Determination for the Populations used in the Molecular Analysis

Flow cytometry was used to determine the ploidy level of each individual from the six populations included in the molecular analysis. The fluorescence intensity of 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei was analyzed with a Partec PA II flow cytometer. The ploidy level of each plant was determined using samples of fresh leaf tissue following the recommendations of the Partec P kit (CyStain UV precise P;

Partec). 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 2 min incubation, samples were filtered through a 50 μm nylon mesh directly into the sample tube, to which 1.5 mL DAPI stain solution (Partec P Kit CySatin UV) was added. The mixture was incubated for another 2 min at room temperature and analyzed. 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.

The ploidy level determination for morphological characterizations was previously performed by Urbani et al. (2002).

Statistical Analyses

Inter simple sequence repeats products were scored for the presence (1) and absence (0) of homologous DNA bands. The GENALEX 6 software (Peakall and Smouse, 2006) was used to determine the number of polymorphic loci for each population. Principal coordinates analysis was performed to estimate the diversity within and among populations. Polymorphism within populations was also quantified using Shannon's information index (Shannon and Weaver, 1949). The proportion of distinguishable genotypes per population was determined using Genotype and Genodive software (Meirmans and Van Tienderen, 2004) to detect possible mutation events. This software can analyze data from polyploids, especially asexually reproducing groups, allowing the user to eliminate as far as possible the contribution of mutations to the estimation of recombinant genetic diversity (Meirmans and Van Tienderen, 2004).

Means of each trait and population, coefficients of variability, and mean separations using the Tukey test were calculated using InfoStat 1.1 software (Di Rienzo et al., 2002). A multivariate ANOVA and principal component analysis were performed with the morphological data to evaluate the existing variability among and with populations using InfoStat 1.1 software. The relation between the morphological and molecular data was evaluated using the Pearson's correlation coefficient. Analysis of molecular variance was used to partition the total molecular variance within and among populations using GENALEX 6 (Peakall and Smouse, 2006) based on 999 permutations.

RESULTS

Morphological and Agronomic Characterization

Variability among and within populations of *P. simplex* was observed for most of the 23 evaluated traits (Table 2) with the exception of winter survival. Biomass yield was the most variable trait considering differences among and within populations. This variability was observed across the growing season considering the four harvests. Length of the basal raceme was also a highly variable trait, but most of this variation was observed within rather than among populations. In contrast, important diversity was observed for leaf hairiness and presence of a terminal raceme, but most of this diversity was present among rather than within populations. All evaluated plants were able to tolerate the winter temperatures and regrowth during the following spring. In addition, tiller length and stigma color were low variable traits.

When the diversity for the 23 traits was evaluated based on the multivariate data analysis, it was possible to observe that 57% of the variation was present among populations and 43% within populations. The minimum genetic distance was observed between the 2*x* and 4*x* populations collected in Villa Ángela (Table 3). The between-population diversity was similar among the other populations included in this study. When the diversity within populations was considered, the most variable populations were diploid (Table 4). More than 50% of the total variability was contained in these two 2*x* populations from Los Gatos and Villa Ángela (Table 4). Among the 4*x* populations, the one sympatric to the 2*x* population from Villa Ángela was the most variable, explaining 15.4% of the total variation.

Molecular Characterization

A new group of plants was generated using seed gathered at each location with the objective of accomplishing the molecular analysis. The ploidy level of each of the 90 plants was determined using flow cytometry and it was coincident with the previously observed levels. Moreover, 60% of the new plants from Villa Ángela were tetraploid, and the other 40% were diploid.

One hundred forty loci were evaluated from which 138 ended up being polymorphic (Table 1). Considering the total variation observed, 70% was present among populations and 30% within populations. The lowest inter-population diversity was observed between the 2*x* and 4*x* populations from Villa Ángela (Table 5; Fig. 2).

The percentage of polymorphic loci was higher in the diploid populations (Fig. 3). Furthermore, each individual 2*x* plant was identified as a different and unique genotype (Fig. 3). Considering the percentages of polymorphic loci and genotypes per 4*x* population, it was possible to observe that the population from Villa Ángela was the most variable (Fig. 3) while the populations from Reconquista and Pirané were the less variable ones. Furthermore, 60% of the plants from Mercedes were identified as a single genotype. The predominance of a single genotype was also observed for the other two allopatric populations, that is, 93 and 100% of the populations from Pirané and Reconquista, respectively, belonged to one genotype. In contrast, the most abundant genotype represented only 33% of the 4*x* population from Villa Ángela.

DISCUSSION

Currently, it is well known that most apomictic species are highly diverse. However, the sources of the observed diversity are not well defined for most species. The genus

Table 2. Variation observed for 23 traits among and within six populations of *Paspalum simplex*.

Traits	Population											
	Los Gatos 2*x*		Villa Ángela 2*x*		Villa Ángela 4*x*		Reconquista 4*x*		Mercedes 4*x*		Pirané 4*x*	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Biomass 1, g	308 a†	46	401.1 a	57.3	549.6 b	37.2	562.5 b	39.3	603.8 bc	31.9	708.9 c	46.6
Biomass 2, g	70 b	40	98.5 c	58	120.3 c	50	117 c	46	116.2 c	43.4	41 a	46.5
Biomass 3, g	125.5 a	49.2	191.5 b	44	234.4 c	38.5	144 a	41.3	188.2 b	29.6	194.5 b	34
Biomass 4, g	120.4 a	59.6	163.9 b	42	275.3 d	35.3	216.4 c	33	244.6 cd	30.9	240.5 cd	35.9
Length of basal raceme, cm	7 cd	22.8	6.6 bc	15.4	6.4 b	14.7	7.4 d	15.2	5.3 a	19.2	6.2 b	23.2
Length of mid raceme, cm	4.9 bc	24.5	4.3 a	17	4.8 ab	17.8	5.3 c	18.3	4.4 ab	19.4	4.5 ab	27.4
Length of upper raceme, cm	2.8 ab	45.3	2.9 bc	33.3	2.9 bc	48.8	3.4 c	34.7	2.3 a	37.1	2.4 ab	36.3
Leaf length, cm	24.8 ab	25.9	22.5 a	23	27 b	26.4	22.1 a	28.1	22.6 a	18.7	24.2 ab	24.3
Leaf width, cm	1.3 cd	25	1.2 bc	21.3	1.4 d	20.5	1.4 cd	21.5	1.1 ab	15.6	1.1 a	18.3
Number of racemes	22.1 b	31.5	22.1 b	20.7	20.4 b	24	16.1 a	19.3	21.1 b	31.9	20 b	16.7
Inflorescence length, cm	22.6 b	16.5	20.7 a	20.7	21.3 ab	16.7	20.1 a	14.7	21 a	12.4	21.2 ab	16.2
Tiller length, cm	120.2 a	10.35	122 a	10	131.8 b	10.3	132.5 b	11.4	131.1 b	9.8	139.6 c	7.2
	Observed variation											
Anther color	80% purple and 20% light purple		90% light purple and 10% purple		90% light purple and 10% purple		95% purple and 5% light purple		100% purple		85% light purple and 15% yellow	
Anthocyanin in spikelet	90% abundant and 10% medium		80% medium and 20% absent		95% medium and 5% absent		95% medium and 5% abundant		100% medium		100% abundant	
Flowering beginning	15 Oct.–1 Jan.		15 Sept.–1 Jan.		1 Sept.–15 Dec.		1 Sept.–15 Nov.		1 Sept.–1 Nov.		1 Oct.–1 Dec.	
Flowering ending	1–15 Jan.		1–15 Jan.		1 Nov.–15 Jan.		1 Nov.–15 Dec.		1–15 Nov.		15 Dec.	
Flowering peak	1–15 Mar.		1 Mar.–15 Apr.		1 Mar.–15 Apr.		1 Mar.– 15 Apr.		15 Apr.		15 Apr.	
Growth habit	50% semierect, 30% postrate, and 20% erect		60% semierect, 30% erect, and 10% postrate		95% semierect and 5% erect		95% semierect and 5% erect		100% semierect		80% semierect and 20% postrate	
Leaf blade hairiness	50% scarce, 30% absent, and 20% medium		50% absent, 30% scarce, and 20% medium		63% scarce, 28% medium, 4% absent, and 4% abundant		100% absent		100% absent		100% absent	
Leaf sheath hairiness	40% medium, 30% absent, and 30% scarce		50% scarce, 40% medium, and 10% abundant		55% abundant, 15% medium, and 30% scarce		100% absent		100% absent		45% absent, 40% scarce, and 15% medium	
Stigma color	93% purple and 7% light purple		100% purple		100% purple		100% purple		100% purple		100% purple	
Terminal raceme	60% present and 40% absent		80% absent and 20% present		75% absent and 25% present		100% absent		100% present		100% absent	
Winter survival	100% alive		100% alive		100% alive		100% alive		100% alive		100% alive	

†Means within rows followed by different letters are significantly different.

Table 3. Euclidean genetic distances among populations of *Paspalum simplex* estimated based on phenotypic characteristics.

Populations	Euclidean genetic distance					
	Los Gatos 2*x*	Villa Ángela 2*x*	Villa Ángela 4*x*	Reconquista 4*x*	Mercedes 4*x*	Pirané 4*x*
Los Gatos 2*x*	0.00					
Villa Ángela 2*x*	1.29	0.00				
Villa Ángela 4*x*	1.40	0.94	0.00			
Reconquista 4*x*	1.70	1.51	1.51	0.00		
Mercedes 4*x*	1.60	1.24	1.38	1.31	0.00	
Pirané 4*x*	1.49	1.43	1.39	1.20	1.67	0.00

Table 4. Variation patterns of six populations of *Paspalum simplex*, estimated by means of multivariate analysis of variance.

Population	*F* modified of Wilks	Total variance (%)
Los Gatos 2*x*	5.69	30.8
Villa Ángela 2*x*	3.99	21.6
Villa Ángela 4*x*	2.85	15.4
Reconquista 4*x*	2.30	12.4
Mercedes 4*x*	1.93	10.4
Pirané 4*x*	1.72	9.3

Paspalum is proposed as a model for evolutionary studies in species sharing this type of asexual reproduction. *Paspalum simplex* is a good representative species of the genus since it contains 2*x*, 3*x*, 4*x* and 6*x* cytotypes, and apomixis always appears linked to polyploidy (Urbani et al., 2002). This research has focus on studying the diversity present among and within 2*x*, 4*x*, and mixed 2*x*–4*x* populations of *P. simplex* with the aim of contributing to a better understanding of how new apomictic genotypes are generated in the genus.

Two different techniques were used for estimating the diversity present in populations of *P. simplex*. One of them was a characterization based on morphology, phenology, and agronomic performance of individual plants while the other was a molecular characterization based on ISSR markers. Both techniques used in this research have similar results considering the diversity observed among populations ($r = 0.6$) as well as within populations ($r = 0.9$). The observed similarity is an indication of the efficiency of both approaches for assessing diversity in plant species, and it also allows for a better and stronger interpretation of the obtained results.

The results indicated that the greatest diversity in *P. simplex* is present in diploid populations that are located in the western part of its natural distribution. This finding is expected to be directly related to the mode of reproduction of the species since Espinoza and Quarin (1997) have indicated that the diploid cytotype of *P. simplex* is sexual and cross-pollinated. In contrast, tetraploid populations are low variable and a single genotype predominates as the result of the apomictic mode of reproduction. However, the 4*x* population sympatric to a diploid population was highly variable and shared most of its diversity

Table 5. Pairwise population matrix of Nei's genetic distance and Shannon's information index (Shannon and Weaver, 1949) of population of *Paspalum simplex*.

	Nei's genetic distance						Shannon's information index	SE
Populations	Los Gatos 2*x*	Villa Ángela 2*x*	Villa Ángela 4*x*	Reconquista 4*x*	Mercedes 4*x*	Pirané 4*x*		
Los Gatos 2*x*	0.000						0.206	0.020
Villa Ángela 2*x*	0.074	0.000					0.160	0.020
Villa Ángela 4*x*	0.110	0.030	0.000				0.118	0.018
Reconquista 4*x*	0.194	0.244	0.261	0.000			0.015	0.006
Mercedes 4*x*	0.166	0.153	0.167	0.208	0.000		0.099	0.018
Pirané 4*x*	0.168	0.194	0.248	0.225	0.244	0.000	0.015	0.005

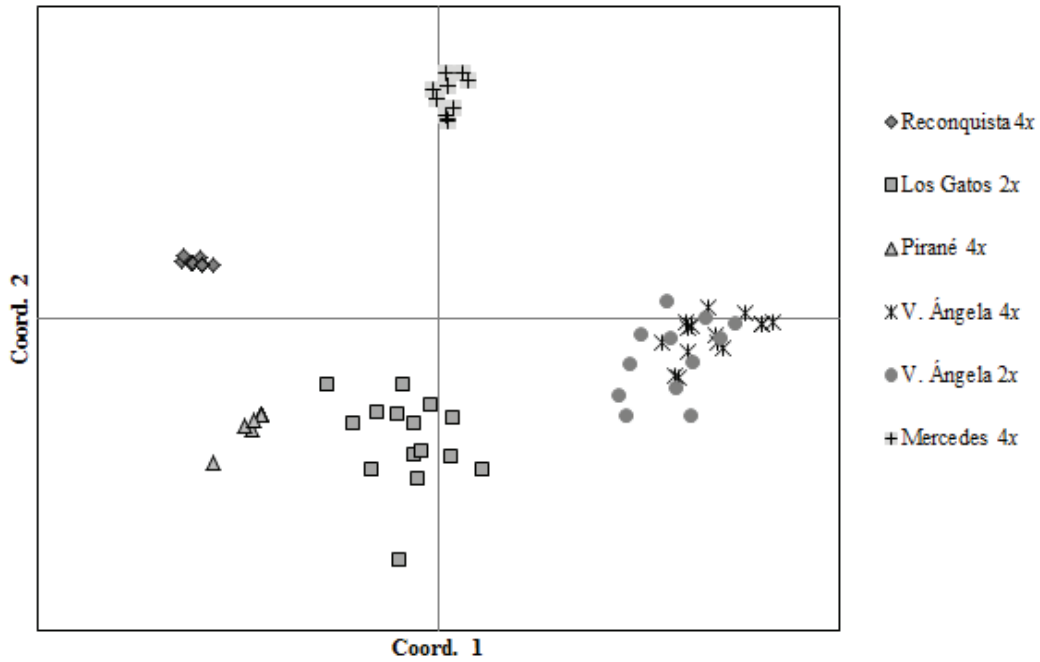

Figure 2. Principal coordinates (Coord.) graphic of natural populations of *Paspalum simplex*. V. Ángela, Villa Ángela.

with the intermixed 2*x* population. This observation is an indication of gene flow between ploidy levels. Hörandl and Paun (2007) have indicated that the observed pattern of diversity among apomictic populations being allopatric and sympatric to sexual ones is common to other genera, such as *Taraxacum* and *Antennaria*. These authors attributed most of the diversity observed in apomictic-sympatric populations to backcrossing with sexual relatives. Moreover, new tetraploid plants could be the result of crosses between 2*x* and 4*x* plants with 2*x* plants forming unreduced gametes. This has already been observed for other two species of *Paspalum*, that is, *Paspalum cromyorhizon* Trin. ex Döll (Quarin et al., 1984) and *Paspalum rufum* Nees ex Steud. (Norrmann et al., 1994). Although a few plants were obtained by making several thousand crosses between 2*x* and 4*x* plants, they were all tetraploid. In addition, 3*x* plants have also been obtained with low reproductive efficiency from crosses between 2*x* and 4*x* plants in *P. notatum* (Burton and Hanna, 1992), *Paspalum intermedium* Munro ex Morong & Britton, and *Paspalum quarinii* Morrone & Zuloaga (Norrmann et al, 1994, as *Paspalum brunneum* auct. non Mez).

Quarin et al. (1989) have described another possible interaction between sexual and apomictic populations, which involves a unidirectional gene flow from diploid to tetraploid populations. The idea is based on (i) the generation of apomictic-3*x* genotypes when an unreduced gamete of a diploid plant is fertilized by a reduced gamete from a different genotype and (ii) the formation of new 4*x* plants through the fertilization of an unreduced gamete, originated by apospory, of the 3*x* plant by a reduced gamete from a diploid plant. There is strong evidence in *Paspalum* supporting this idea, starting by the regular occurrence of apomictic 3*x* plants in multiploid species (Gates et al., 2004). In addition, Siena et al. (2008) have reported that low expression of apomixis occurs in 2*x* plants and that 3*x* plants are generated by 2*x* × 2*x* crosses, when an occasional unreduced gamete of aposporous origin is fertilized by a reduced male gamete. Moreover, Quarin et al. (1989) observed high proportion of artificial 4*x* hybrids that are generated by 3*x* × 2*x* crosses in *P. quarinii*, *P. notatum*, and *P. intermedium*. This phenomenon is explained by the high fertility present in plants with odd ploidy levels that reproduce by apomixis and by the regular formation of B_{III} hybrids in aposporous plants. Triploid plants have been reported for several species of *Paspalum* (Quarin, 1992). Although they are not as common as 4*x* and 2*x* plants they have been found growing in 2*x* populations. In the particular case of *P. simplex*, Urbani et al. (2002) reported the occurrence of 3*x* plants in natural populations. Furthermore, a 3*x* plant was found in the mixed 2*x*–4*x* population from Villa Ángela included in this research. In addition, 6*x* plants have also been found in almost pure 4*x* populations of *P. simplex* (Urbani et al., 2002). These 6*x* plants are suspected to be B_{III} hybrids generated from 4*x* genotypes.

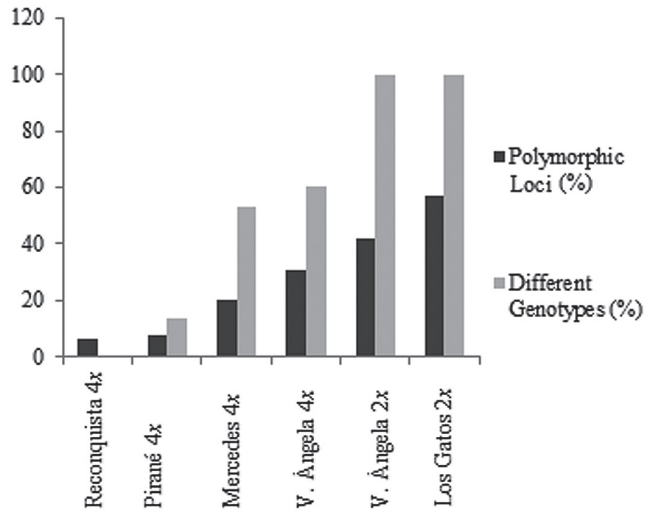

Figure 3. Proportion of polymorphic loci and different genotypes in six populations of *Paspalum simplex*. V. Ángela, Villa Ángela.

Greater diversity in 4*x*-sympatric populations was also observed in *P. notatum*, a species that also includes different ploidy levels and apomixis is linked to polyploidy (Daurelio et al., 2004). The evidence generated for *P. notatum* and *P. simplex* indicates that the genotypic variability among most apomictic species of *Paspalum* is generated in sexual 2*x* populations that behave as cross-pollinated. Tetraploid genotypes are then created from these 2*x* populations, where 3*x* plants probably play an important role. In addition, new tetraploid genotypes could then be the result from crosses between 2*x* and 4*x* plants living in proximity. These new 4*x* apomicts are then able to colonize the different environments around the area where 2*x* populations are located.

Of special interest considering the possibility of collecting and selecting the best adapted genotypes for cultivation as forage crops is the diversity present among 4*x* allopatric populations. The intrapopulation diversity was not the same among the three 4*x* allopatric populations included in this study, that is, the population from Mercedes was more diverse than the others. These differences might be related to the age of the populations, but it can also be related with a higher rate of residual sexuality occurring in the most variable population. Further research including a higher number of 4*x* allopatric populations is necessary for better understanding the development of new clonal populations or ecotypes. In addition, the important diversity observed for traits of agronomic interest, that is, growth habit, reproductive phase, and biomass yields, is indicating the feasibility of initiating a breeding program for *P. simplex* with the aim of generating forage cultivars for the subtropical beef-cattle (a mixture of *Bos taurus*, *Bos indicus*, and interspecific hybrids) industry. Although biomass-yield data is preliminary because it was taken only twice during each of the two evaluated growing seasons,

this information has contributed to the estimation of the diversity present in this species, and also it has allowed to identify genotypes with greater capacity to grow in the environment where the evaluation took place.

In conclusion, diversity in *P. simplex* is generated in outbreeder-2*x* populations, and polyploidization and fixation of novel 4*x* genotypes by apomixis is crucial for the observed diversity in 4*x* populations. The greatest diversity present among 4*x* populations relates to the fact that a well-adapted genotype predominates in every isolated-4*x* population. The great diversity observed for specific morphological, phenological, and agronomic traits indicates that ecotype selection can be used to identify superior forage types in *P. simplex*.

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