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ORIGINAL ARTICLE

Patterns of genetic diversity in natural populations of *Paspalum* agamic complexes

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Abstract *Paspalum* has many multiploid species displaying a wide range of ploidy levels and reproductive systems including apomixis. However, not much is known about the genetic structure of natural populations of the apomictic species of *Paspalum*. The aim of this work was to evaluate the genetic diversity of several natural populations belonging to five species of *Paspalum*. A total of 13 populations were analyzed using amplified fragment length polymorphism (AFLP). The AFLP data revealed maximal genotypic diversity and significant levels of genetic diversity in diploid and mixed diploid–tetraploid populations of *P. denticulatum* and *P. rufum*, where all individuals represent different genotypes. This may be mainly due to the reproductive system of diploid members and the gene flow from diploids to polyploids. The pure populations of tetraploids consist of either multiple genotypes (*P. nicorae*) or of one dominant genotype with a few deviated genotypes (*P. denticulatum* and *P. lividum*). Here, the main source of variability may be the residual sexuality, which continues generating new genotypic combinations. The hexaploid populations of *P. buckleyanum* consist of a single AFLP genotype and each population represents a particular genotype suggesting that populations arose from independent polyploidization events. This study represents one of the first reports of genetic diversity in natural populations of several *Paspalum* agamic complexes. Apomixis in these five species may be acting as a successful method for the dispersion of better adapted genotypes.

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Introduction

Agamic complexes have been described as units in which sexual ecotypes and apomictic counterparts are found at different ploidy levels, in most cases diploid and tetraploid, respectively. Furthermore, apomicts with higher ploidy levels also exist (Savidan 2000). In nature, these complexes can set a variety of populations in relation to the genetic system of their members. Thus, it is possible to find pure populations composed exclusively of individuals with only one ploidy level, or mixed populations, where individuals with different ploidy levels coexist. Both types of communities have been described for different agamic complexes such as *Taraxacum* (den Nijs and Menken 1996), *Capillipedium–Dichantium–Bothriochloa* (De Wet and Harlan 1970), *Panicum maximum* (Pernès 1975), *Tripsacum* (Moreno-Perez et al. 2009), *Ranunculus auricomus* (Hörandl et al. 1997; Hörandl and Greilhuber 2002; Cosendai and Hörandl 2010) and *Paspalum* (Norrmann et al. 1989; Urbani et al. 2002; Daurelio et al. 2004). The fact that these communities exhibit a multiplicity of combinations between ploidy levels and reproductive systems involving apomixis suggests that this type of asexual reproduction does not necessarily imply uniformity within a species, and that diversity could be maintained in populations (Hörandl and Paun 2007).

Several studies on the genetic structure of apomictic populations have identified a general pattern of diversity, where it is most partitioned among populations and to a lesser degree within populations as in autogamous plants (Palacios and Gonzalez-Candelas 1997; Carino and

Daehler 1999; Van der Hulst et al. 2000; van Dijk 2003; Houliston and Chapman 2004; Hörandl 2006; Paun et al. 2006; Majeský et al. 2012). However, in apomictic polyploids of *Amelanchier* (Campbell et al. 1999), *Taraxacum* (Van der Hulst et al. 2003), *Ranunculus* (Paun et al. 2006) and *Crataegus* (Lo et al. 2009), high levels of variability have also been observed. Particularly in *Paspalum*, the studies of genetic diversity of sympatric and allopatric populations from *P. notatum* showed that an apomictic tetraploid population sympatric to sexual diploids exhibit higher levels of diversity compared to tetraploid allopatric populations (Daurelio et al. 2004).

The patterns of genetic diversity in natural populations have usually been determined using morphological, ecological, cytological, and biochemical (isoenzymes) traits. However, polymerase chain reaction (PCR)-derived markers obtained with no species specific primers have become remarkably popular since they do not require sequence information for the target species (Nybom 2004). In this context, AFLP constitute valuable informative traits since they do not require any prior knowledge about the genome and a large number of markers are easily and quickly available (Gaudeul et al. 2004). Comparison of different molecular markers employed to assess intraspecific diversity in plants, including dominant (RAPD, AFLP, ISSR) and codominant (STMS) markers, have demonstrated that estimations made with dominant markers are useful and could be directly comparable (Gaudeul et al. 2004; Nybom 2004; Garoia et al. 2007). Moreover, some authors suggest that AFLP markers are more useful than SSRs to discriminate individuals from neighbouring populations on a small geographical scale, and also to assign correctly the percentage of individuals in their population of origin, and recommend the use of AFLP to population genetic studies (Campbell et al. 2003; Gaudeul et al. 2004; Garoia et al. 2007). In contrast, AFLPs are less recommended to be used in studies concerning heterozygosity (Gaudeul et al. 2004).

Paspalum is a large genus of the grass family (Gramineae) displaying a wide range of ploidy levels and reproductive systems. A large proportion of taxonomic entities are multiploid species which constitute agamic complexes with different chromosome races (cytotypes). In these complexes, diploid cytotypes are mostly sexual and self-incompatible. Nevertheless embryological evidences of apospory have been reported in several of these diploids, indicating some potential for apomixis (Quarin et al. 1982; Norrmann et al. 1989). Moreover, functional aposporous embryo sacs have been reported for diploid *P. rufum* (Siena et al. 2008). Co-specific polyploid cytotypes (mainly tetraploids) are apomictic, pseudogamous, and self-compatible (Quarin 1992). Usually, the different chromosome races that constitute these agamic complexes are morphologically very similar and routine cytological studies are required to determine their ploidy levels (Quarin and Lombardo 1986; Quarin and Norrmann 1987; Norrmann et al. 1989; Quarin 1992). Moreover, multiploid species are the rule in the genus *Paspalum* and autoploidy seems to be the most likely origin of these polyploid series. There are cytological evidences that most apomictic multiploid species of *Paspalum* originated by autoploidy (Bennett and Bashaw 1966; Norrmann et al. 1989; Quarin et al. 1998; Hojsgaard et al. 2008). In addition, the typical polysomic inheritance of autopolyploids has been documented at least in two of these species: *P. simplex* (Pupilli et al. 1997) and *P. notatum* (Stein et al. 2004).

In particular, *P. buckleyanum* Vasey (synonym: *P. alcalinum* Mez) is a multiploid species with sexual diploid and apomictic polyploid cytotypes. Burson (1997) analyzed three accessions from different localities in Paraguay: a sexual self-fertile diploid, a facultative apomictic tetraploid, and an apomictic pentaploid. Sartor et al. (2011) analyzed several individuals from distant populations from Argentina and Paraguay. Three of them were purely hexaploid, and one was a mixture of apomictic pentaploid and hexaploid individuals including one exceptional heptaploid plant.

Paspalum denticulatum Trin. is also a multiploid species that grows in northern Argentina, western Brazil, and Paraguay. Sexual diploid, apomictic triploid and facultative apomictic tetraploid cytotypes have been described for this species (Quarin and Burson 1991; Morrone et al. 2006; Sede et al. 2010; Sartor et al. 2011). Quarin and Burson (1991) suggest autopolyploidy as the most likely origin of the tetraploid cytotype, based on the high frequency of quadrivalents and other multivalent associations observed at meiosis.

Paspalum lividum Trin. has a distribution that extends from the southern United States and Mexico southward to Argentina. Several cyto-embryological studies have revealed that this species is tetraploid and reproduces by apomixis (Gould 1958, 1968; Reeder 1967; Burson and Bennett 1971; Davidse and Pohl 1972; Quarín 1977; Pagliarini et al. 2001). However, there is a previous formal record of heptaploidy for an occasional accession ($2n = 7x = 70$) (Snyder 1953).

Paspalum nicorae Parodi, a valuable forage grass, spreads across Brazil, Paraguay, Uruguay and Argentina. It has been described as a pseudogamous apomictic tetraploid species (Burson and Bennett 1970). Then, a large number of tetraploid accessions were reported by different authors (Moraes Fernandes et al. 1974; Pagliarini et al. 2001), including a list of more than 50 introductions collected throughout the state of Rio Grande do Sul, Brazil (Reis et al. 2010).

Paspalum rufum is a perennial grass distributed through low wet areas and swamps of Brazil, Paraguay, Uruguay

Table 1 Identification and collection sites of thirteen populations of five *Paspalum* species

Species	Population	Locality of collection
P. buckleyanum	B2	Argentina. Salta, El Galpón
	B3	Argentina. Chaco, Antequeras
	B4	Paraguay. 120 km NW of Asunción
P. denticulatum	D1	Argentina. Chaco, Tres Isletas
	D2	Argentina. Chaco, Antequeras
	D3	Paraguay. 52 km NW of Asunción
P. rufum	R1	Argentina. Corrientes, Paso Rosario
	R3	Argentina. Santa Fé, Gdor. Crespo
	R2	Argentina. Chaco, 50 km N of Resistencia
P. nicorae	N1	Argentina. Corrientes, Santa Ana
	N2	Argentina. Santa Fé, Cayastá
	N3	Argentina. Corrientes, Mantilla
P. lividum	L1	Argentina. Salta, El Galpón

and Argentina. This species involves sexual diploid and facultative apomictic autotetraploid cytotypes (Norrmann et al. 1989; Quarin et al. 1998; Siena et al. 2008). Moreover, natural populations may be constituted by a single cytotype or may be composed by different cytotypes (Sartor et al. 2011).

Since *Paspalum* has many multiploid species, and their apomictic polyploid cytotypes are likely autopolyploid, it is relevant to know the genetic variability among and within populations composed either by one or several ploidy levels.

The objective of this study was to evaluate the genotypic and genetic variability in several natural populations belonging to five different species of *Paspalum* using AFLP.

Materials and methods

Plant material

Thirteen populations involving five species of *Paspalum* were collected from a wide range of their natural geographic distribution in Argentina and two localities in Paraguay (Table 1; Fig. 1a, b). Populations were sampled by collecting rhizome pieces from single plants. These samples were collected at least 10 m apart from each other to ensure that genotype frequencies were not biased. A minimum of 10 and a maximum of 90 individual cuttings were sampled per population, though collections ranged from 50 to 90 individuals for most populations. The ploidy level and mode of reproduction in each population was previously determined by flow cytometric analysis (Sartor et al. 2011). The ploidy level was determined for each individual and the mode of reproduction was evaluated in 6–10 individuals per population randomly selected. Between 20 and 30 individuals per population were selected for the AFLP analysis. In those populations where more than one cytotype were found, the proportion of individuals analyzed for each ploidy level was equivalent to the proportion observed in the field.

DNA isolation and AFLP studies

Genomic DNA was extracted from 4 g of fresh leaves by using the method of Dellaporta et al. (1983) including the modifications introduced by Ortiz et al. (1997). Sample quality was checked by measuring Abs. 260 nm/Abs. 280 nm index and by agarose 1 % gels, to confirm DNA integrity and absence of RNA contamination. The AFLP procedure was undertaken following the manufacturer's instructions of the AFLP Analysis System I (Life Technologies, Invitrogen) with minor modifications. About 1 µg of genomic DNA was simultaneously digested with *Eco*RI and *Mse*I. The restricted genomic DNA fragments were ligated to *Eco*RI and *Mse*I adapters and constituted the template for pre-amplification. Pre-amplification products were diluted (1:10) with 1× TE buffer and used as templates for selective amplification. UNO Biometra thermocycler was used for both pre-amplification and selective amplification. Following amplification, the PCR products were mixed with 2 µL of loading dye (98 % formamide, 10 mM EDTA, 0.025 % bromophenol blue and 0.025 % xylene cyanol), denatured at 95 °C for 5 min and immediately placed on ice. Six microlitres of the denatured samples were loaded onto denaturing 6 % polyacrylamide gels and electrophoresis was conducted by applying a constant power of 60 W at a temperature of 50 °C for 2 h in a Scie-Plas electrophoresis cell connected to a Consort/EV233 power supply. Amplification products were visualized by using the Silver Staining-System from Promega and digitized using hp scanjet 4670.

Fifteen combinations of oligonucleotides were tested in a subsample of two individuals from each population. Of these, the primers combinations *Eco*RI-ACC/*Mse*I-AAA, *Eco*RI-AGC/*Mse*I-AAA were selected for their capacity to detect informative polymorphisms. These primer combination yielded a total number of bands comprised between 114 and 205 (depending on the population, Table 2) when used to analyze the totality of thirteen populations. An extra primer combination (*Eco*RI-ACT/*Mse*I-AGC) was assayed in the populations of *Paspalum buckleyanum*. The percentage of polymorphism was calculated for each population and considering individuals with the same ploidy level.

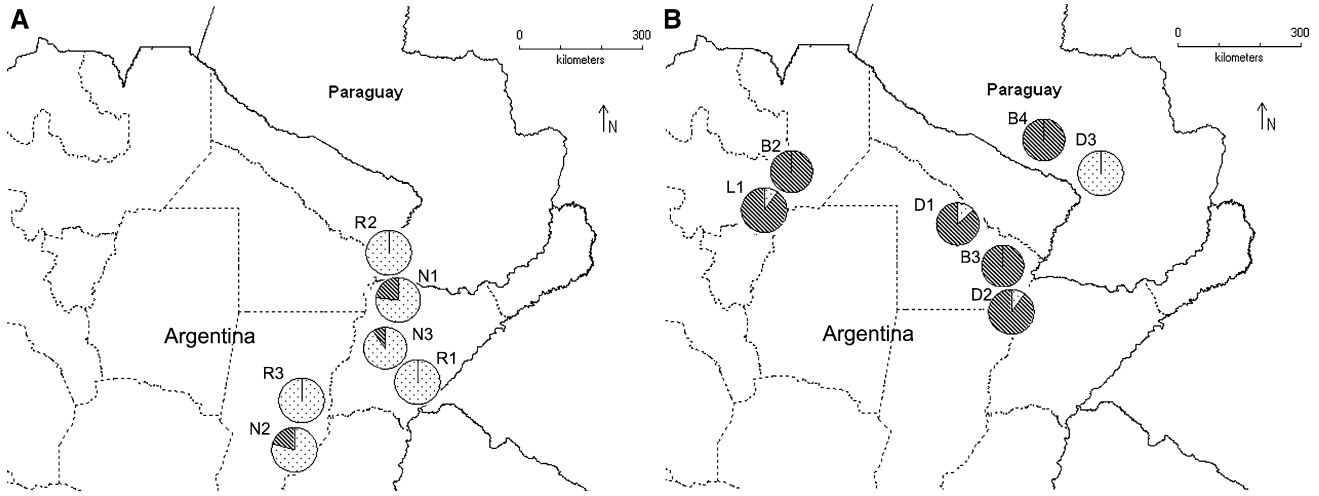

Fig. 1 Site location and frequency of AFLP genotypes of the 13 populations of *Paspalum* used in this study. **a** *P. nicorae* (N1, N2 and N3), *P. rufum* (R1, R3 and R6); **b** *P. buckleyanum* (B2, B3 and B4), *P. denticulatum* (D1, D2 and D3), *P. lividum* (L1). The *striped* section of pie charts represents individuals with the same genotype in each population; the *dotted* section represents individuals with different genotypes

Data analysis

Markers were scored from the gels with the aid of a light box for presence (1) or absence (0) of bands and were included in a binary matrix by using the Microsoft Excel program. Only clear and unambiguous DNA bands were analyzed. A blank space was used to denote missing data caused by a failure in amplification or the presence of unclear/poorly defined bands. A band was considered polymorphic only if it was present in a least one genotype and absent in the others.

Matrices were used for the determination of Nei's genetic distance by applying GenAlEx program (Peakall and Smouse 2006). Based on genetic distance, analyses of principal coordinates (PCoA) were made for each population. In addition, the total number of fragments and the percentage of polymorphic fragments over the total fragments analyzed were also determined. The differentiation among populations and cytotypes within population was made by determining the number of private alleles (alleles or markers present in a cytotype or population and absent in the others) and by analyzing the molecular variance (AMOVA) with a significant test based on 999 permutations. In the AMOVA the genetic divergence was calculated using the PhipPt value, a multiallelic analogue of the F_{ST} which is called the coefficient of gene differentiation (Nei 1973). Comparison of diversity between cytotypes was made in all populations with more than one ploidy level. Analysis of variability between populations was made to *Paspalum buckleyanum*, *P. denticulatum* and *P. nicorae* because they represent three different genetic systems within *Paspalum.*

Genotypic variation was evaluated using the software GENOTYPE/GENODIVE (Meirmans and Van Tienderen 2004) which allows the analysis of data from polyploids, especially asexually reproducing groups (Paun et al. 2006). GENOTYPE assigns genotypic identity to individuals using data from the genetic markers and GENODIVE calculates and tests indices of clonal diversity, such as the number of genotypes (G), Nei's (1987) diversity index (D), the corresponding evenness (E), and the Shannon–Wiener diversity index (H') (Shannon and Wiener 1949). D values range from 0 (for uniclonal population) to 1 (in those population where each individual has a different genotype). Evenness (E) ranges from 0 in a population where all individuals represent different genotypes or where one genotype dominates and the others are represented by a single individual, to 1 in population where all clones are represented by the same number of individuals (Paun et al. 2006). Shannon–Wiener (H') is one of the most commonly index used in ecology to represent the overall diversity (Meirmans 2006). Estimates of genotypic and genetic variability were performed for the 13 populations separately.

According to Paun et al. (2006), GENOTYPE/GENODIVE (Meirmans and Van Tienderen 2004) was used to detect possible mutation events. User-specified thresholds (in the infinite allele mutation model, IAM corresponding to numbers of allowed mutations) were used in the GENOTYPE program to assign clone mates with small genetic differences to the same asexual lineage (having a within 'genotype' diversity higher than 0). After that, different indices of clonal diversity were calculated (G, D, E and H') for different threshold values, in order to

Table 2 Score of markers obtained from the AFLP amplification and estimates of genetic/genotypic variation for thirteen populations belonging to five species of *Paspalum*

Species and populations	Ploidy level	Mode of reproduction[a]	Genetic diversity indices				Genotypic diversity indices				
			TB	PB	%PB	PA	*N*	*G*	*D*	*E*	*H*′
P. buckleyanum											
B2	6*x*	Apo	201	0	0	16	30	1	0	1	0
B3	6*x*	Apo	198	0	0	29	30	1	0	1	0
B4	6*x*	Apo	205	0	0	8	30	1	0	1	0
P. denticulatum											
D1	4*x*	Fac Apo	122	35	29	10	30	4	0.51	0.50	0.37
D2	4*x*	Fac Apo	114	34	30	6	30	3	0.13	0.38	0.13
D3	2*x*	Sex	197	131	66	30	23	23	1	1	1.36
	3*x*	Apo	197	17	9	1	2	2	1	1	0.30
	4*x*	Fac Apo	197	75	38	6	5	5	1	1	0.70
P. rufum											
R1	4*x*	Apo	134	91	68	na[c]	29	29	1	1	1.46
R2	2*x*	Sex	130	130	100	na	30	30	1	1	1.48
R3	2*x*	Sex	139	82	59	1	8	8	1	1	0.90
	4*x*	Apo	139	126	91	21	21	21	1	1	1.32
P. nicorae											
N1	4*x*	Fac Apo	122	96	79	0	30	23	0.97	0.70	1.28
N2	4*x*	Fac Apo	146	132	90	1	29	23	0.98	0.78	1.32
N3	4*x*	Fac Apo	118	90	76	0	30	28	0.99	0.88	1.41
P. lividum											
L1	4*x*	Fac Apo	168	27	16	na	20	14	0.94	0.65	1.07
L1′[b]	4*x*	Fac Apo	168	27	16	na	20	2	0.34	0.74	0.22

TB total number of bands considered, *PB* number of polymorphic bands, *%PB* percentage of polymorphic bands, *PA* number of private alleles, *N* number of individual analyzed, *G* number of genotypes, *D* genotype diversity, *E* genotypic evenness, *H*′ Shannon–Wiener diversity index, *Apo* apomictic, *Fac Apo* facultative apomictic, *Sex* sexual

[a] Mode of reproduction determined by flow cytometry (Sartor et al. 2011)

[b] Values of genetic and genotype diversity within L1 population after three allowed mutations using de infinite allele model (IAM)

[c] Not analyzed

eliminate as far as possible the contribution of mutations to the estimation of recombinant genetic diversity (Paun et al. 2006).

Results

Genetic variability in *Paspalum* species comprised of pure and mixed populations

Three populations were analyzed for *Paspalum denticulatum*: D1 and D2, comprising of tetraploid facultative apomicts and D3, comprising of 2*x*, 3*x* and 4*x* cytotypes, where diploids are fully sexual, triploids fully apomictic and tetraploids facultative apomicts. D1 exhibited 4 different genotypes, while D2 showed only 3 multilocus genotypes. These few genotypes represented between 30 and 93 % of individuals in both populations and the percentage of polymorphisms reached to 29 % in D1 and 30 % in D2. Although the number of genotypes found in these populations was similar, the values of both genotypic diversity indices *D* and *E* were higher in D1 than in D2 because the former showed two predominant genotypes while the latter showed the same genotype in 28 of the 30 individuals tested (Table 2). The third population (D3) composed of diploid, triploid and tetraploid individuals showed the highest genotypic variation for *P. denticulatum*, with values of *D* and *E* equal to 1, which means that even the small number of 3*x* and 4*x* detected in this populations represent different genotypes. The maximum percentage of polymorphic bands as well as the highest value of diversity was found among 2*x* individuals (66 % and $H' = 1.36$). Analysis of molecular variance (AMOVA) indicated that in the mixed population D3 the 80 % of variability occurred within each cytotype, with only a 20 % of variability among ploidy levels (Table 3). Principal

Table 3 Analysis of molecular variance (AMOVA) for AFLP data in *Paspalum* species

	df[a]	Sum of squares	Variance	Percentage of variation	PhiPt[b]
P. denticulatum all populations					
Among populations	2	163.572	11.351	45	0.454
Within populations	34	465.050	13.678	55	
P. denticulatum, D3 population					
Among cytotypes	2	101.851	5.282	20	0.202
Within cytotypes	27	562.083	20.818	80	
P. rufum, R3 population					
Among cytotypes	1	49.503	2.471	11	0.106
Within cytoptyes	27	563.493	20.870	89	
P. nicorae, all populations					
Among populations	2	59.082	1.085	29	0.295
Within populations	72	186.903	2.596	71	

[a] Degree of freedom

[b] Genetic structure indices (all $P < 0.001$)

coordinate analysis based on genetic diversity allowed the distinction of the populations D1, D2 and D3 in three well differentiated groups, and none of the genotypes was found repeated in two different populations (Fig. 2). The AMOVA reveal that 55 % of the variation occurred within populations, while the remaining 45 % corresponded to the variation among populations.

In *Paspalum rufum* variability analysis involved a sexual diploid population (R2) and two populations that were mixtures of sexual diploid and apomictic tetraploid individuals (R1 and R3). Unfortunately, only tetraploid individuals were available for analysis in population R1 because the only diploid individual initially collected in this population has not survived under cultivation. The three populations showed maximum genotypic diversity, with *D* and *E* equal to 1, which means that all individuals analyzed correspond to a particular genotype (Table 2). All genotypes were also discriminated with the PCoA. Pure diploid population (R2) presented the highest percentage of polymorphic bands (100 %) and one of the highest values of genetic diversity ($H' = 1.48$). These values are expected for a population composed of individuals with a reproductive system involving sexuality and cross-pollination. In mixed population R1 the values of genetic diversity were also high (PB = 68 %, $H' = 1.46$). In mixed population R3 diploid genotypes exhibited lower percentage of polymorphic bands and genetic diversity than those obtained for tetraploid genotypes from the same population (Table 2). Moreover, the number of private alleles was also higher in the 4*x* level, probably due to the small sample of diploid category, which could overlook the number of polymorphic bands. Similarly to the mixed population of *P. denticulatum* (D3), the AMOVA indicated that most part of variability in mixed population R3 occurred within each cytotype (89 %), while the remaining variability (11 %) corresponded to variability between 2*x* and 4*x* cytotypes.

Genetic variability in *Paspalum* species comprised of exclusively pure tetraploid apomictic populations

Three pure facultative apomictic tetraploid populations were analyzed for *Paspalum nicorae*, N1, N2 and N3 with important values of genotypic variation. While *D* ranged from 0.97 to 0.99, the values of *E* varied from 0.65 to 0.88 indicating that most individuals of these populations corresponded to different multilocus genotypes, which was also corroborated with the PCoA. In addition, the percentages of polymorphic bands for N1, N2 and N3 were 79, 90, and 76 %, respectively, and the values of Shannon–Wiener index ranged from 1.28 (N3) to 1.41 (N2). These records constitute extraordinarily elevated levels of polymorphism for an apomictic tetraploid species that showed only 5 % of residual sexuality (Sartor et al. 2011). The AMOVA attribute a low percentage (29 %) of variation among populations, suggesting low differentiation among communities. Supporting this proposal, only one private allele was found in N2 population (Table 3). The other alleles analyzed were scored in more than one population.

In the case of *Paspalum lividum*, twenty individuals were selected from facultative apomictic tetraploid population L1. In this population, PCoA allowed to identify 14 different genotypes (Fig. 3). Despite the high genotypic diversity indices that were observed ($D = 0.94$ and $H' = 1.07$), the percentage of polymorphic bands was only 16 % indicating a low differentiation between genotypes.

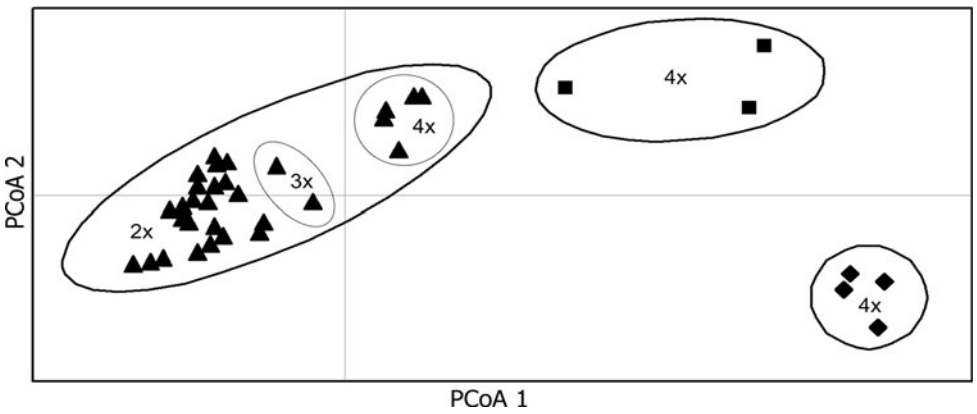

Fig. 2 Principal coordinates analysis based on genetic distance within and between populations D1 (*filled diamonds*), D2 (*filled squares*) and D3 (*filled triangles*) of *P. denticulatum*. Ploidy level of each population is included in the figure

Genetic variability in *Paspalum* species comprised exclusively pure hexaploid apomictic populations

The three analyzed populations of *Paspalum buckleyanum* (B2, B3 and B4) were homogeneously composed of apomictic hexaploid individuals. In each population, a single multilocus genotype was identified ($G = 1$) by the GENODIVE program and the PCoA, meaning that all individuals from the same population were genotypically identical (Fig. 1b). This clonal structure is also evidenced in the total lack of multilocus genotype diversity ($D = 0$ and $H' = 0$). The analysis of the diversity partition between populations showed a total diversity of $D = 0.67$, which is exclusively due to genetic difference among the genotypes of the three populations ($G'_{st} = 1$) (Table 4). Each genotype also exhibited alleles that were genotype-specific, which reinforces the hypothesis of a differentiation between populations.

Influence of mutation in the genetic diversity

The analysis of mutational influence in genetic diversity was performed for each one of the 13 populations involving the 5 *Paspalum* species studied: *P. denticulatum* (D1, D2 and D3), *P. buckleyanum* (B2, B3 and B4), *P. rufum* (R1, R2 and R3), *P. nicorae* (N1, N2 and N3) and *P. lividum* (L1). The results indicated that the number of genotypes remained unchanged for 12 populations. In L1 population, the number of genotypes decreased to a stable minimum after 3 mutational steps in the infinite allele model (IAM), by changing from 14 to 2 genotypes with a *D* value decreasing from 0.93 to 0.33 (Table 2). In agreement with this result, the PCoA made to L1 population of *P. lividum* showed that the 14 genotypes detected to this population constituted two well-differentiated groups (Fig. 3).

Discussion

The analysis of genetic and genotypic variability in 13 populations belonging to 5 multiploid species of *Paspalum* showed heterogeneous results, which may be grouped in three different genetic systems.

Genetic variability in *Paspalum* species which develop populations comprised of pure and mixed cytotypes

This system congregates *P. denticulatum* and *P. rufum*, both represented by populations with one cytotype or populations with mixed cytotypes. Particularly *P. denticulatum* can be considered a paradigmatic species within the genus, as it presents the typical genetic structure of multiploid species with sexual and apomictic cytotypes (Quarin 1992). Pure 4*x* populations D1 and D2, previously classified as facultative apomictic by Sartor et al. 2011, were composed of a few different genotypes and relatively low genetic variability in comparison with the cytotype-mixed population D3. In D3, sexual diploid plants are predominant and coexist with a few apomictic triploids and facultative apomictic tetraploids (Sartor et al. 2011). Tetraploid individuals in this population showed high levels of variability and, unlike pure populations D1 and D2, each of the five plants analyzed represents a different genotype. In the case of *P. rufum*, diploid (R2) and diploid–tetraploid mixed (R3) populations exhibited higher levels of genetic diversity and similar levels of genotypic diversity than those observed in tetraploid pure population (R1). However, the levels of variability of R1 were greater than in pure tetraploid populations D1 and D2. In R1, one diploid individual had been initially detected by flow cytometry (Sartor et al. 2011). Unfortunately, the diploid plant died soon after collection and despite of a further collection attempt no other diploid plant was found in this population. It might be suspected that this diploid plant arose by dihaploidy from tetraploids. Dihaploidization has been reported in many apomictic taxa, including genera of the grass family. Diploid–tetraploid–dihaploid cycles have been considered a key natural strategy to generate and maintain genetic variability in the genus *Dichanthium* (de Wet 1968; de Wet and Harlan 1970) and in *Panicum*

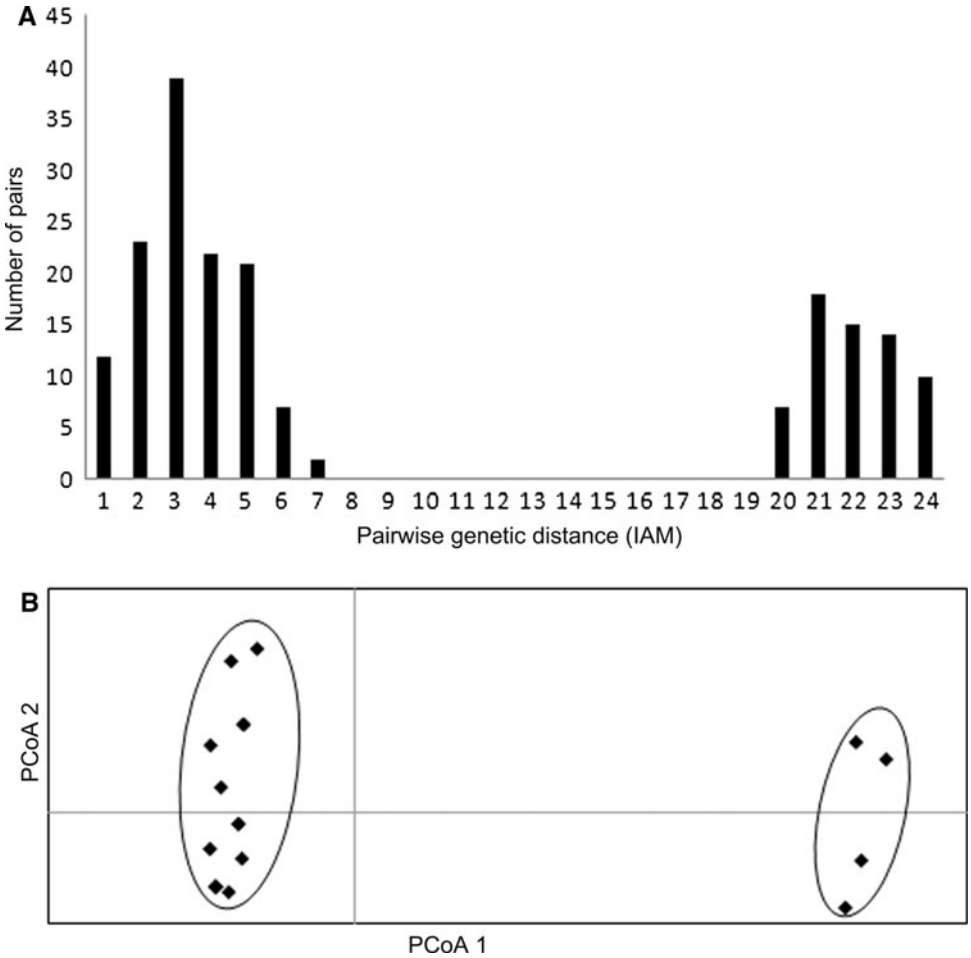

Fig. 3 Genetic diversity of L1 population of *Paspalum lividum*. Frequency distribution of pairwise distances between individuals calculated with GENOTYPE (**a**) and principal coordinates analysis based on genetic distance within L1 population (**b**)

Table 4 Analysis of partitioning of diversity in natural populations of *Paspalum buckleyanum*

Partitioning of diversity	Genotypic diversity	Shannon–Wiener	Corrected Shannon–Wiener
Total diversity	0.67	0.48	0.48
Average diversity within populations	0.00	0.00	0.00
Fraction of among populations diversity (G_{st})	1.00	1.00	1.00
Fraction of among populations diversity, corrected (G'_{st})	1.00	1.00	1.00

maximum (Savidan and Pernès 1982). However, a dihaploidization event has never been reported for species of the genus *Paspalum*, a genus in which different ploidy levels seem to be connected by recurrent polyploidization, but not by diploid–tetraploid–dihaploid cycles (Siena et al. 2008). Thus, the collected tetraploids of R1 would be in contact with rare diploids.

In general, grasses with sexual and out-crossing reproduction, like diploid *P. denticulatum* and *P. rufum*, have higher levels of genetic variability within populations because of cross-pollination among different genotypes (Huff et al. 1993). At the same time, these diploids may contribute to the variability at polyploid level. It was demonstrated that diploid plants of *P. rufum* may produce, beside the normal sexual embryo sac, some unreduced embryo sacs (Norrmann et al. 1989). These aposporous sacs are functional and may be fertilized by a reduced male gamete to produce triploid plants ($2x + x = 3x$) (Siena et al. 2008). These triploids may in turn lead to new tetraploids when an unreduced female gamete of the triploid is fertilized by a reduced male gamete from a diploid plant ($3x + x = 4x$). Moreover, in mixed populations (diploid + tetraploid), a $2n$ ($2x$) gamete from an aposporous sac generated in diploid plants may eventually be fertilized by a reduced gamete ($n = 2x$) of a tetraploid plant, originating a new tetraploid genotype (Norrmann et al. 1994; Siena et al. 2008). Siena et al. (2008) also demonstrated that in $2x$–$4x$ mixed populations, recurrent polyploidization may take place through fertilization of unreduced gametes of diploid plants rather than through fertilization with unreduced male gametes. Thus, the levels of genetic and genotypic variability in diploid and mixed populations of *P. denticulatum* and *P. rufum* may be mainly due to the

reproductive system of diploid members and the gene flow from diploids to polyploids. According to Yamauchi et al. (2004) and Kao (2007) these new polyploids could have been expanded establishing pure populations through adaptive advantage provided by the condition of apomictic polyploid.

In the case of *P. denticulatum*, tetraploid pure populations exhibit low levels of genetic diversity, with a predominance of one or two clones. Low levels of diversity within populations may be a consequence of the establishment of populations by seeds from some better adapted individuals (Van Dijk 2003), where apomixis restricts the possibilities to generate new genotypes through hybridization (Lo et al. 2009). Otherwise, the 4*x* individuals of populations D1 and D2 are facultative apomictic, and conserve the capacity to produce new genotypes by recombination (Sartor et al. 2011). Therefore, residual sexuality can play a crucial role to generate genotypic variation in apomictic populations, because it has the greatest quantitative effects in the short term (Hörandl and Paun 2007). This same pattern of genotype distribution is frequently observed in populations of apomictic complexes (Gornall 1999; Hörandl et al. 2001; Daurelio et al. 2004; Lo et al. 2009).

It has been proposed that within apomictic populations of the same species, genotypic diversity is partitioned mainly among populations and to a lesser degree within populations (Stebbins 1950; Nybom 2004; Majeský et al. 2012). Our studies demonstrate that the total variability among the three populations of *P. denticulatum* is equally generated by intra- and inter-population variation. Thus, these communities may be still in a differentiation process.

Genetic variability in *Paspalum* species comprised of exclusively pure tetraploid apomictic populations

This second genetic system is represented by *Paspalum nicorae* and *P. lividum*. Unlike the previous species, only apomictic tetraploid populations were found in these two species. Surprisingly, high levels of genotypic and genetic diversity were detected in tetraploid populations of *P. nicorae* and *P. lividum*. High levels of genetic diversity have also been reported for other polyploid apomictic genera: *Taraxacum* (Van der Hulst et al. 2003), *Amelanchier* (Campbell et al. 1999), *Ranunculus* (Paun et al. 2006) and *Crataegus* (Lo et al. 2009). One of the sources of variation within population may be the formation of novel genotypes through allopolyploidization and introgressive hybridization (Van Dijk 2003; Hörandl 2006; Hörandl and Paun 2007; Nybom 2007; Lo et al. 2009). However, diploid individuals have never been found for *P. nicorae* and *P. lividum* (Moraes Fernandes et al. 1974; Pagliarini et al. 2001; Reis et al. 2010; Sartor et al. 2011). The absence of diploid plants eliminates the possibility that new polyploidization processes are occurring within populations. Lo et al. (2009) suggest that high levels of genetic variability in apomictic polyploid populations may be attributed to occasional sexual recombination. The fact that *P. nicorae* populations N1, N2 and N3 and *P. lividum* population L1 were classified as facultative apomictics (Sartor et al. 2011), suggests that the principal source of variability within populations is the residual sexuality, which continues generating new genotypic combinations (D'Souza et al. 2004; Hörandl and Paun 2007; Nybom 2007; Whitton et al. 2008; Lo et al. 2009). Another source of genetic variability in natural populations, especially in apomictic populations are mutations (Hörandl and Paun 2007; Nybom 2007). Such mutations can be accumulated over time, leading to the establishment of a network of clone mates (Paun et al. 2006; Hörandl and Paun 2007; Majeský et al. 2012). The influence of mutations in the genetic variability detected for *P. nicorae* may be discarded since the analyses made with the GENOTYPE/GENODIVE program demonstrated that the number of genotypes do not varied by adding mutational steps. However, mutation analysis have revealed that the better part of the genetic variation observed in the population L1 of *P. lividum* is due to mutations, since the number of genotypes was reduced from 14 to 2 and the real level of variability was also reduced when the IAM model added three mutational steps. Thus, L1 may be an ancient community with a clonal origin, where the effects of variability-generating forces such as mutations can be currently observed.

In contrast with the high levels of genetic diversity observed within populations, low levels of genetic diversity were detected between the three *P. nicorae* populations N1, N2 and N3. This low differentiation among populations may imply gene exchange among populations due to pollen flow through floral visitors or seed dispersion by birds and small mammals (Courtney and Manzur 1985; Guitián 1998; Lo et al. 2009). Lo et al. (2009) also proposed that the lack of geographical structure could be caused to the presence of similar funders for both neighboring and distant populations. These founder genotypes can be maintained and multiplied in each population, maintaining a similar composition among them (Lo et al. 2009).

Genetic variability in *Paspalum* species comprised of exclusively pure hexaploid apomictic populations

Paspalum buckleyanum corresponds to a third genetic system. The three populations of *P. buckleyanum* collected for this analysis were hexaploid and approximately 200 bands of AFLP markers were analyzed for each population. A single and specific band pattern was detected for each population. Monomorphic bands were observed among

individuals of the same population, indicating that each population was constituted of a single genotype. It is important to point out that uniclonal populations were rarely found in studies involving high-resolution molecular markers. In *Ranunculus carpaticola*, uniclonal 6*x* populations have been detected using AFLP markers (Paun et al. 2006). These authors suggest that in 6*x* populations of *R. carpaticola* the lack of variability is due to: (1) the fact that they are newly formed communities, (2) there is no sexual events occurrence or there is a strong selection pressure against genotypes that may be caused by residual sexuality. These mechanisms may be acting in 6*x* apomictic populations of *P. buckleyanum*, which were classified as apomictic without residual sexuality (Sartor et al. 2011). The hexaploids populations of *P. buckleyanum* may have arisen from tetraploid through fertilization of unreduced female gametes with reduced pollen generating B_{III} $(2n + n)$ descendants. In turn, these new hexaploid genotypes may have been successful in competing with their tetraploid ancestors and other species to rapidly colonize available habitats through apomictic reproduction. This mechanism of polyploidization is a phenomenon that occurs in 4*x* races of several species of *Paspalum* (Quarin and Hanna 1980; Urbani et al. 2002; Rebozzio et al. 2011; Sartor et al. 2011). There are previous cyto-embryological studies that demonstrated the existence of tetraploids in *P. buckleyanum* (Burson 1997); however, 4*x* individuals were not found in the populations studied in this work, so it is unknown whether these tetraploids were able to produce B_{III} offspring. The tetraploid accession PI 404638 reported by Burson (1997) was collected 120 km northwest of Asunción, Paraguay. In 2008, visiting that place we found a large population from which we sampled 35 individual plants. Surprisingly, all those individuals were hexaploid (Sartor et al. 2011) and thirty of them were taken for the present study (population B4). Moreover, we have participated in several collection trips through northeast and central-north Argentina to western Paraguay and we failed to collect tetraploid *P. buckleyanum,* although we usually found the hexaploid and occasionally the pentaploid cytotypes (data not shown). These findings indicated that hexaploidy and obligate apomixis constitute the most successful current combination for this multiploid species.

Conclusion

This study represents one of the first reports of genetic diversity in natural populations of several *Paspalum* agamic complexes. The species studied in this work have developed different and efficient mechanisms of adaptability and surviving efficiency. Although apomixis is a strong component present in most of the species analyzed, it does not necessarily imply lack of variability in natural populations. Their genetic systems and reproductive modes sustain the generation of new genotypic combinations mainly through recurrent polyploidization events and residual sexuality and to a lesser extent through mutations. The polyploidization system in multiploid species implies: the existence of sexual allogamous diploid cytotypes, which occasionally develop unreduced aposporous embryo sacs, and the consequent formation of apomictic triploids, which can be acting as a bridge in the recurrent polyploidization through $2n + n$ triploid $\times$ diploid, or $2n + n$ in diploid $\times$ tetraploid hybridizations. Otherwise, polyploidization in one step can occur and a new apomictic tetraploid genotype can be generated if an unreduced $2n$ gamete of a diploid plant is fertilized by a male haploid gamete from a neighbouring apomictic tetraploid plant $(2n)$. Higher levels of ploidy can arise through different pathways. For example, pentaploids may arise from a sexual event concerning an aposporous embryo sac of an apomictic tetraploid and reduced pollen from a diploid donor $(2n + n = 5x)$, or from an occasional sexual event involving a sexual embryo sac of a facultative apomictic tetraploid and a reduced pollen from a hexaploid $(n + n = 5x)$. Hexaploids in turn may arise from apomictic tetraploids by $2n + n$ fertilization. Our results suggest that recurrent polyploidization and residual sexuality ensure genetic diversity in apomictic populations.

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