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Genetic variability in natural populations of *Paspalum dilatatum* Poir. analyzed by means of morphological traits and molecular markers

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Abstract Native species show adaptive traits that are difficult to find in introduced species. The Pampas region in Argentina is a valuable nature reserve of grasses and *Paspalum dilatatum* Poir. is one of the most important grasses found there. Based on ploidy level and on morphological traits, five biotypes of *P. dilatatum* have been described. Two of them were included in this study: a tetraploid biotype with sexual reproduction and a pentaploid biotype with apomictic reproduction. We analyzed the genetic diversity in eight native populations from the Salado basin, Argentina, using both quantitative traits and molecular data (RAPD) with these aims: to obtain information of the degree of phenotypic variation in that area, to know which the pattern of distribution of this variation is and to look for association between molecular markers with populational or biotypic differentiation. Cluster analysis based on morphological data grouped the individuals of the different populations by ploidy level. Molecular markers showed the inverse situation because individuals were grouped by geographic origin as opposed to biotype. Moreover, since RAPD did not discriminate between biotypes with sexual or apomictic reproduction, they are probably not associated with mating system. The results let us conclude that polygenic traits such as LP, LBSR, NRT and NSP can discriminate between biotypes and molecular markers such as bands 12, 40, 19 and 46 can be used to discriminate among populations, probably because they detect neutral variation.

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Introduction

Paspalum dilatatum Poir. (dallisgrass) is an important summer forage grass in South America, whose place of origin includes Uruguay, South Brazil and Argentina (Holt 1956). This grass is a perennial and rhizomatous plant with smooth leaves and a strong root system and a panicle with 3–5 spikes (Burkart 1969). Dallisgrass starts to

grow in early spring, before most warm season grasses, and continues until late fall (Holt 1956).

Because of the phylogeny of this genus is not well defined yet, taxonomic categories like ''section'' or other have not been assigned so far (Zuloaga, personal communication). In this way *P. dilatatum* is grouping into the informal group *Dilatata*, which includes other species clearly related such as, *P. urvillei* Steudel, *P. pauciciliatum* Hertel and *P. dasypleurum* Kuntze (Barreto 1974). The genus *Paspalum* includes many polyploid species, with a basic chromosome number of 10 (Quarín and Norrmann 1986). Based on ploidy level and on morphological traits, five biotypes of *P. dilatatum* have been described. The biotype ''Flavescens'' is most often found in southern Brazil, Uruguay and Argentina (Casa 1995). It is tetraploid ($2n = 4x = 40$) with sexual reproduction, predominantly autogamous and have yellow anthers. The biotype ''Common'' predominates in the province of Buenos Aires, Argentina. It is pentaploid ($2n = 5x = 50$), has apomictic reproduction, purple anthers and the highest forage quality of the biotypes. The reproductive system of these two biotypes was analyzed by Bashaw and Holt (1958).

The suitability of the pentaploid biotype as a forage grass is due to its adaptability to different soils, its perennial growth and its high dry matter yield production during summer (Carámbula 1982). The last character allows this species to fill the deficit in forage production during warm season (Pahlen 1986). However, its potential use as forage is reduced, at least in part, due to high susceptibility to the common biotype to the fungus *Claviceps paspali* (Burson et al. 1973). Infection by *C. paspali* results in the production of ergot, a sticky material, toxic to livestock if consumed in large quantities. The efficient use of this natural resource can only be optimized if its diversity is evaluated and quantified in some way.

Three other biotypes of *P. dilatatum* have also been described and are known as ''Torres'', ''Uruguaiana'' and ''Uruguayan''. These biotypes are hexaploid ($2n = 6x = 60$), present different meiotic behavior and occur in different zones of South America. Burson et al. (1991) described these biotypes as pseudogamous and suggested that they developed aposporic apomictic embryonic sacs.

The origin of the tetraploid and pentaploid biotypes was established by means of cytological studies. The diploid species *P. juergensii* Hackel (genome JJ) and *P. intermedium* Mourung (genome II) have been proposed as the likely genome donors of the yellow anther biotype (genome IIJJ) (Burson and Bennett 1972; Burson et al. 1973). Recently, Casa (1995) suggested, based on data from molecular markers studies, that *P. intermedium* and *P. jurgensii* are not the progenitors of *P. dilatatum* and proposed that future studies should include other *Paspalum* species from the *Quadrifaria* and *Paniculata* groups as potential donors of the II and JJ genomes, respectively. Also, slightly different forms of II genome were observed in four other diploid species of the Quadrifaria group: *P. quadrifarium Nees*, *P. brunneum Poir*, *P. haumanii Parodi* and *P. densum Poir*. (Espinoza and Quarín 2000). Burson (1991) suggested that the pentaploid biotype was originated from cross between the tetraploid and hexaploid ''Uruguayan'' biotype.

Morphological traits, in particular quantitative traits, have been used in these species to characterize and to assess the variability of natural populations (Percival and Couchman 1979; Pahlen 1986; García et al. 2002).

Molecular biology has allowed the development of rapid, sensitive, and specific screening methods to study genetic diversity. Molecular techniques like restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPDs) have been used in the genus *Paspalum* to study, for instance, genetic diversity (Liu et al. 1994; M'Ribu and Hilu 1996; Jarret et al. 1998; Pereira et al. 2000), the phylogenetic relationship among closely related species of this genus (Pereira et al. 1999; Casa 1995) and to analyze the mode of reproduction (Ortiz et al. 1997)

In the studied area, the populations of this species show a predominance of apomictic biotypes. Therefore, these populations, like populations of the autogamous biotypes, might show reduced variability within population and wide diversity among populations. In this work we analyze the genetic diversity in eight native

populations of *P. dilatatum* from the Salado basin province of Buenos Aires, Argentina, using both quantitative traits and molecular data. The objectives of this work were: to obtain information of the degree of phenotypic variation in that area, to know which the pattern of distribution of this variation is and to look for association between molecular markers with populational or biotypic differentiation.

Materials and methods

Populations of *P. dilatatum* collected from eight different locations of the Salado river basin in the province of Buenos Aires (Argentina) were analyzed (Fig. 1). Around 20 individuals of each population were included in the quantitative traits analysis and 8 individuals as representatives of each population in the molecular analysis (Table 1). No tetraploid genotypes were found in the populations of Brandsen and Pereyra Iraola while in the rest of the populations both tetraploid and pentaploid biotypes were found. The ploidy level was established by anther color.

Soil characteristics and a lack of natural drainage explain why that the principal movement of water is vertical in the Salado river basin. Together with topographic variation these characteristics
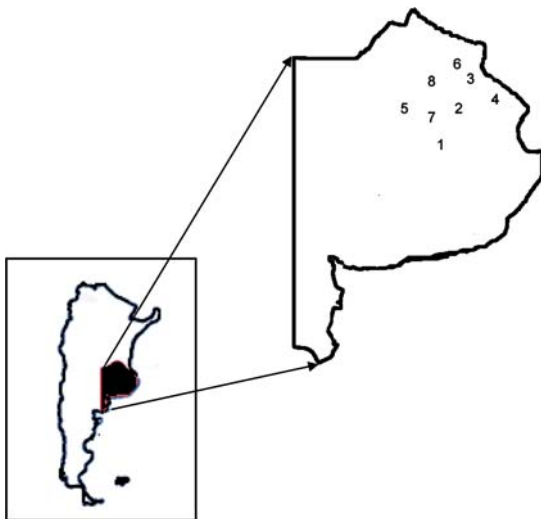

Fig. 1 Map showing the location of 8 population of *Paspalum dilatatum*. 1, Belgrano; 2, Brandsen; 3, La Plata; 4, Magdalena; 5, Monte; 6, Pereyra Iraola; 7, Ranchos; 8, San Vicente

define heterogeneous vegetation (F. Grecco and V. Rossi 1997) in a region with a warm and wet climate.

Samples were taken from isolated plants (separated by at least 3 m) to avoid sampling the same individual twice. Plants were kept in plastic pots with sandy soil and controlled irrigation.

Quantitative characters

Diversity at quantitative traits was evaluated by means of six reproductive and six vegetative characters of high forage value. The quantitative characters measured are resumed in Table 2. All the characters were measured on two reproductive tillers from each genotype. Because the characters LFL, WFL, LL$_2$ and WL$_2$ were correlated, two new characters: foliar area of the flag leaf and foliar area of the second leaf (FAFL and FAL$_2$) were calculated as measurement of the surface area. These characters and %SC were only used in multivariate analysis.

Another character included in the analysis was Resistance to *Claviceps paspali* (R*Cp*). It was determined by direct observation on two panicles per plant. Four levels of attack were considered: (1) Panicle without fungus, (2) 30% of infected panicle, (3) 60% of the panicle infected and (4) Panicle totally attacked.

RAPD markers

DNA isolation

Five hundred milligram of fresh leaf tissue was cut in small pieces, and transferred to 1.5 ml Eppendorf tubes with three beads of glass (3 mm diameter). Tubes were submerged in liquid nitrogen, vortexed for 1 min to grind the tissue and then DNA was isolated by adding 1 ml of extraction buffer Rapid One Step Extraction (ROSE) (Steiner et al. 1995), washed twice with phenol and once with chloroform. Finally, DNA was precipitated with 1 vol of isoamylalcohol and 10% 3 M Sodium Acetate. The DNA quality was estimated by measuring the OD (optical density) at 260/280 nm in a spectrophotometer (Shimadzu Model UV-160A).

Table 1 Collection sites of *Paspalum dilatatum* Poir. and number of plants analysed in each ploidy level

Population	Genotypes analysed				Location
	Quantitative traits		Molecular data		
	$2n = 4x$	$2n = 5x$	$2n = 4x$	$2n = 5x$	
Belgrano	5	14	1	7	35°51′ LS, 58°35′ LW
Brandsen	–	19	–	8	35°17′ LS, 58°13′ LW
La Plata	1	14	1	7	34°54′ LS, 57°55′ LW
Magdalena	6	14	4	4	35°00′ LS, 57°41′ LW
Monte	6	14	1	7	35°22′ LS, 58°48′ LW
Pereyra Iraola	–	19	–	8	34°52′ LS, 58°08′ LW
Ranchos	2	17	2	6	35°26′ LS, 58°15′ LW
San Vicente	12	9	4	4	35°03′ LS, 58°30′ LW

Table 2 Quantitative characters measured

Quantitative characters	
Vegetative characters	Reproductive characters
Number of Nodes per Tiller (NNT)	Number of Reproductive Tillers (NRT)
Tiller Length (TL)	Length of Panicle (LP)
Length of Flag Leaf (LFL)	Length of Basal Spike Rachis (LBSR)
Width of Flag Leaf (WFL)	Number of Spikes per Panicle (NSP)
Length of 2nd Leaf (LL$_2$)	Number of Spikelets per Panicle (NSpP)
Width of 2nd Leaf (WL$_2$)	Percentage of Spikelets with Caryopse (%SC)*

* This character was registered by puncture

Polymerase chain reaction

RAPD reactions were carried out in a final volume of 15 ul. About fifteen ng of genomic template DNA plus 10× reaction buffer (50 mM KCl, 10 mM Tris–HCl, pH 9.0; 1% Triton X 100), 7.5 mg BSA (bovine serum albumin, Sigma Chemical Corporation), 12 ng of primer (DNA-gency, Malvern USA), 0.2 mM of each dNTPs (Promega Biotech Corporation), 1.5 mM MgCl$_2$, and 1 U *Taq* DNA polymerase (*Highway Mol Biol*-InBio-UNICEN, Tandil, BA, Arg) completed the reaction mixture. A thermocycler (Thermolyne Temp-Tronic®) was programmed as follows: an initial denaturing step of 5 min at 94°C, 40 cycles consisting each of a denaturing step of 45 s at 94°C, 60 s at 36°C, and 60 s at 72°C with a final step of 7 min at 72°C. Gels were then photographed under UV light with a Polaroid Camera DS34 and film type 667. The reactions were carried out with low stringency conditions of annealing for standardized the reaction for all the primers tested. Negative controls without template DNA was included in each round of reactions for distinguish between specific and unspecific reaction products.

Twenty-nine primers with sequences that corresponded to different primer series from Operon Tech. Corp. with G-C content between 60–70% were tested (Table 3).

Data analysis

Quantitative characters

Using the collected data, we generated a distance matrix (DM) in the form of column n × row t matrix. The DM was standardized to a zero mean and unity variance to reduce scale effects of the different characters measured. The analysis of the relationship between representative genotypes of the populations was made with a distance coefficient that indicates the genetic distance (GD). The mean taxonomic distance index (MTD) was applied. (Sneath and Sokal 1983). A phenogram was generated by applying the UPGMA (unweighted pair-group method using arithmetic averages) method as the clustering strategy.

The relevance of the characters in the discrimination of individuals was established by means of principal component analysis. This

Table 3 Nucleotide sequence of 10-mer primers used for RAPD genetic similarity analysis of eight natural population of *Paspalum dilatatum* Poir.

Primer	Sequence	Primer	Sequence
A 08	5 ′-ACGCACAACC-3′	OPE 08	5′-TCACCACGGT-3′
OPA 02	5′-TGCCGAGCTG-3′	OPE 09	5′ GGGTAACGCC 3′
OPA 05	5′-AGGGGTCTTG-3′	OPE 15	5′-ACGCACAACC-3′
OPA 07	5′-GAAACGGGTG-3′	OPE 16	5′-GGTGACTGTG-3′
OPA 09	5′-GGGTAACGCC-3′	OPE 18	5 AGGYGACCGT 3′
OPA 10	5′-GTGATCGCAG-3′	OPE 19	5′-ACGGCGTATG-3′
OPA 13	5′-CAGCACCCAC-3′	OPE 20	5′-AACGGTGACC-3′
OPA 14	5′-TCTGTGCTGG-3′	OPF 10	5′-GGAAGCTTGG-3′
OPA 20	5′-GTTGCGATCC-3′	OPG 03	5′-GAGCCCTCCA-3′
OPB 10	5′-CTGCTGGGAC-3′	OPG 05	5′-CTGAGACGGA-3′
OPB 15	5′-GGAGGGTGTT-3′	OPJ 13	5′-CCACACTACC-3′
OPC 04	5′-CCGCATCTAC-3′	OPR 04	5′-CCCGTAGCAC-3′
OPC 11	5′-AAAGCTGCGG-3′	OPW 13	5′-CACAGCGACA-3′
OPC 19	5′-GTTGCCAGCC-3′	OLIGO P 124	5′-ATTGCGTCCGAG-3′
OPC 20	5′-ACTTCGCCAC-3′		

'OP' was derived by primers from operon Technologies, Alameda, CA

analysis departs from correlation matrix. Regression coefficient between the component and the character explains the contribution of each character to each component. The relative significance of characters for the first three components was analyzed.

The means of all characters were calculated for each population and biotype and Tukey's test was applied to contrast population means. Student t-test was used to determine whether differences between biotypes means were statistically significant. Also, variability between biotypes was tested by means of coefficient of variability applying non-parametric Mann–Whitney test (U).

Molecular characters

RAPDs are dominant genetic markers that can not discriminate between homozygotes and heterozygotes (Ferreira and Grattapaglia 1996). In order to generate the DM, bands are analyzed as double state qualitative characters. During the construction of the DM it was assumed that a band strictly identifies one locus with two alleles and bands with different molecular weight correspond to different independent loci (Stewart and Excoffier 1996).

Genetic similarity (GS) was analyzed using one symmetric association coefficient and one asymmetric association coefficient. Simple Matching (SM) was used as symmetric association

coefficient and Jaccard (CAJ) as asymmetric. SM is defined as $SM = (a + d)/ (a + b + c + d)$ and CAJ is defined as $CAJ = a/a + b + c$. Where a represents a band shared by two individuals (positive match), b is a band present in one individual and absent in the other, c is a band absent in one individual and present in the other and d is a band absent in both (negative match) (Sneath and Sokal 1983). Symmetric coefficients consider the double absence of a band whereas asymmetric coefficients ignore it (Legendre and Legendre 1983). A phenogram for each coefficient was generated using UPGMA.

The relative significance of RAPD bands in population discrimination was analyzed by means of principal coordinate component from the similarity matrix. The contribution in the individual discrimination for the first three coordinates was analyzed.

Correlation analysis

Molecular and quantitative data were compared using Mantel correlation test. The correspondence Mantel test was performed to compare the distance and similarity matrices (Smouse et al. 1986). In this test, a correspondence measure (r_{xy}) is calculated between the elements of two matrixes X and Y. A high value of r_{xy} indicates a lineal relation between the elements X_{ij} and Y_{ij} (Beer et al. 1993). Similarity values were transformed in genetic distance values by: GD = 1– (GS).

Results

Quantitative characters

Table 4 shows mean values of the vegetative and reproductive characters.

The means for each population of the morphological characters measured were pairwise compared using the Tukey test, which makes use of the Studentized range (Steel and Torrie 1980). In general there were few significant differences among them. Characters such as LFL, NRT, NSP, LP, LBSR and NSpP can be grouped in only two subsets of means (Table 5).

Brandsen had higher mean values for seven characters, although not statistically different from the other populations. The genotypes from San Vicente were highly heterogeneous and had the lowest mean values of the morphological characters.

In the pentaploid biotype, vegetative and reproductive characters had significant higher values than individuals that were tetraploid, except NRT and NSP (Table 6). %SC, however, was larger in the tetraploid biotype (Table 6).

The coefficient of variability showed that the variation of the vegetative characters in the pentaploid biotype was greater than within the tetraploid biotype, although the difference between biotypes for these characters was not statistically significant (Table 7). The variability in NSpP was significantly greater in the tetraploid biotype and whereas %SC was greater in the pentaploid biotype.

We built a phenogram using the GD distance coefficient from representative genotypes of the different populations (Fig. 2b).

Two main groups can be identified: group A that includes mainly pentaploid individuals and group B consisting of tetraploid individuals. Also, a nucleus was formed by two genotypes from the population collected in Magdalena (Mag 146 and Mag149). Finally, one genotype from Brandsen (Br14) was incorporated in the phenogram. These three genotypes also had extreme values of NSP.

Reproductive characters are relevant in the discrimination of individuals. The variation included in the first component is explained by LP and LBSR. NRT and NSP explained the variation in the second and third component, respectively (Table 8).

The cophenetic correlation coefficient (CCC) was $r = 0.78$ suggesting that the agreement between the distance matrix and the phenogram was high.

Table 4 Means of vegetative and reproductive characters of *Paspalum dilatatum* Poir.

Characters	Populations								Biotypes	
	Belgrano	Brandsen	La Plata	Magdalena	Monte	P.Iraola	Ranchos	San Vicente	Tetraploid	Pentaploid
NNT	3.23	3.26	3.05	3.18	3.12	3.38	3.25	3.07	2.86	3.30
TL (cm)	75.40	74.05	70.07	72.93	74.16	74.99	74.11	70.99	69.23	74.63
LFL (cm)	13.30	15.74	14.40	13.30	12.47	13.56	12.84	13.78	12.57	13.94
WL_2 (cm)	0.55	0.64	0.58	0.56	0.53	0.60	0.55	0.51	0.46	0.59
LL_2 (cm)	17.64	20.02	19.10	18.06	17.26	18.16	16.96	16.96	15.80	18.46
WSL_2 (cm)	0.67	0.76	0.74	0.69	0.68	0.72	0.67	0.64	0.59	0.72
NRT	10.80	8.55	9.30	13.37	10.27	12.61	13.38	13.79	12.40	11.48
NSP	4.86	4.77	4.45	4.78	4.30	4.28	4.59	5.31	4.59	4.60
LP (cm)	13.52	13.68	12.34	12.93	12.86	13.19	13.05	12.29	12.04	13.24
LBSR (cm)	7.12	7.27	6.28	6.66	6.93	7.44	6.99	6.22	5.96	7.14
NSpP	328.43	340.70	312.04	310.19	286.60	317.85	304.38	277.73	242.15	325.97
%SC	14.17	10.70	10.50	14.55	16.11	9.00	9.69	19.63	4.63	3.14

The values were measured by population and by biotype

NNT = number of nodes per tiller; TL = tiller length; LFL = length of the flag leaf; WFL = width of the flag leaf; LL_2 = length of the second leaf; WL_2 = width of the second leaf; NRT = number of reproductive tillers; NSP = number of spikes per panicle; LP = length of the panicle; LBSR = length of the basal spike rachis; NSpP = number of spikelets per panicle; %SC = percentage of spikelets with caryopse

Table 5 Means contrast of vegetative and reproductive characters of *Paspalum dilatatum* Poir., established using Tukey test

	LFL	WFL	WL$_2$	NRT	NSP	LP	LBSR	NSpP	%SC
Belgrano	b	b – c	b – c	a – b	a – b	a	a	a – b	a – b – c
Brandsen	a	a	a	b	a – b	a	a	a	b – c
La Plata	a – b	a – b – c	a – b	a – b	b	b	b	a – b	b – c
Magdalena	b	a – b – c	a – b – c	a – b	a – b	a – b	a – b	a – b	a – b – c
Monte	b	b – c	a – b – c	a – b	b	a – b	a – b	b	a – b
P. Iraola	b	a – b	a – b – c	a – b	b	a – b	a	a – b	b – c
Ranchos	b	b – c	b – c	a – b	b	a – b	a – b	a – b	b – c
San Vicente	a – b	c	c	a	a	b	b	b	a

Means, within a column, followed by the same letters are not significantly different ($P = 0.05$) according to Tukey's test

LFL = length of the flag leaf; WFL = width of the flag leaf; WL$_2$ = width of the second leaf; NRT = number of reproductive tillers; NSP = number of spikes per panicle; LP = length of the panicle; LBSR = length of the basal spike rachis; NSpP = number of spikelets per panicle; %SC = percentage of spikelets with caryopse

Table 6 Student test (*t*) between biotypes characters means of *Paspalum dilatatum* Poir.

	Student *t*
TL	3.72**
LFL	2.85**
WFL	8.12**
LL$_2$	2.86**
WL$_2$	3.93**
NRT	0.84 n.s.
NSP	0.04 n.s.
LBSR	3.57**
NSpP	5.39**
%SC	7.81**

TL = tiller length; LFL = length of the flag leaf; WFL = width of the flag leaf; LL$_2$ = length of the second leaf; WL$_2$ = width of the second leaf; NRT = number of reproductive tillers; NSP = number of spikes per panicle; LBSR = length of the basal spike rachis; NSpP = number of spikelets per panicle; %SC = percentage of spikelets with caryopse

n.s.: Not statistically significant

** Statistically significant at $P = 0.01$ level

Molecular characters

Out of a total of 29 primers only 10 were polymorphic. These 10 primers generated between 4 and 8 bands that varied in size between 250 and 1700 bp with an average size of 730 bp (Tables 9 and 10). We generated a phenogram based on the similarity matrix (Fig. 2a). Only the phenogram obtained from Simple matching association coefficient is showed because the grouping obtained with both coefficients did not show differences. The value of Cophenetic Correlation Coefficients (CCC) was 0.75 suggesting that the level of distortion between the matrix and the phenograms was low.

The phenogram clustered individuals from the Brandsen, Pereyra Iraola, Ranchos, and San Vicente populations by place of origin whereas the clustering of the individuals from the other

Table 7 Coefficient of variability of vegetative and reproductive characters in *Paspalum dilatatum* Poir.

Characters	Biotype		U
	Tetraploid	Pentaploid	
NNT	16.43	17.57	n.s.
TL	15.75	15.02	n.s.
LFL	3.16	3.78	n.s.
WFL	17.39	20.33	n.s.
LL$_2$	4.19	5.23	n.s.
WL$_2$	16.27	18.05	n.s.
NRT	72.74	73.78	n.s.
NSP	25.49	21.95	n.s.
LP	17.19	14.12	n.s.
LBSR	17.61	17.92	n.s.
NSpP	25.90	4.33	*
%SC	24.40	36.49	*

NNT = number of nodes per tiller; TL = tiller length; LFL = length of the flag leaf; WFL = width of the flag leaf; LL$_2$ = length of the second leaf; WL$_2$ = width of the second leaf; NRT = number of reproductive tillers; NSP = number of spikes per panicle; LP = length of the panicle; LBSR = length of the basal spike rachis; NSpP = number of spikelets per panicle; %SC = percentage of spikelets with caryopse

U = Mann–Whitney test

n.s.: not statistically significant

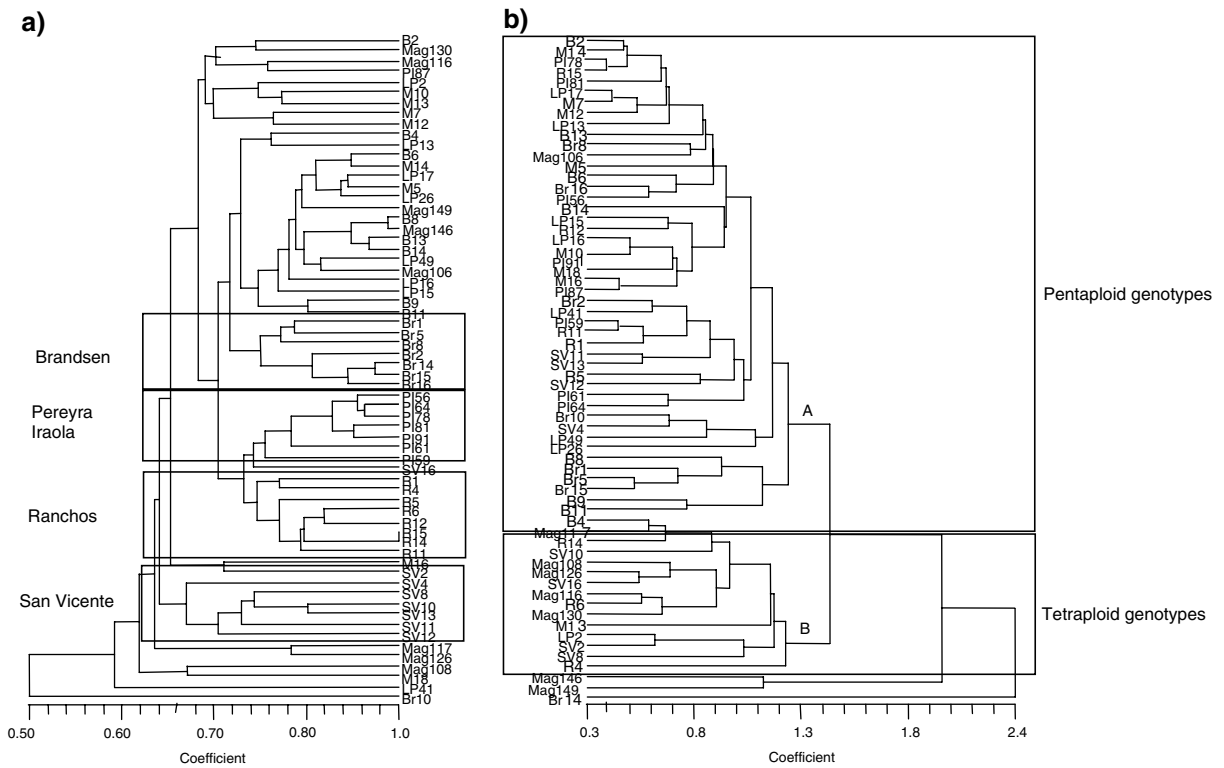* Statistically significant at $P = 0.05$ level

Fig. 2 Cluster analysis phenogram of eight natural population of *Paspalum dilatatum* Poir. (**a**) Phenogram generated from molecular similarity matrix. (**b**) Phenogram based on the morphological distance matrix. B, Belgrano; Br, Brandsen; LP, La Plata; Mag, Magdalena; M, Monte; PI, Pereyra Iraola; R, Ranchos; SV, San Vicente

Table 8 Relative significance of vegetative and reproductive characters of *Paspalum dilatatum* Poir. with respect to the three first principal components

Character	Component 1	Component 2	Component 3
R*Cp*	0.4504	0.5886	0.3906
NNT	0.6887	0.3745	–0.2917
TL	0.6783	–0.2559	–0.0695
FAFL	0.7134	–00.3545	0.3376
FAL$_2$	0.6964	–0.3445	0.3660
NRT	–0.2429	**0.6609**	–0.2818
NSP	0.2553	–0.1761	**–0.6639**
LP	**0.8296**	–0.0296	–0.3760
%SC	–0.4975	–0.5359	–0.2762
NSpP	0.6837	0.1643	0.1363
LBSR	**0.8149**	0.0083	–0.2960

R*Cp*= Resistance to *Claviceps paspali*; NNT = number of nodes per tiller; TL = tiller length; FAFL = foliar area of the flag leaf; FAL$_2$ = foliar area of the second leaf; NRT = number of reproductive tillers; NSP = number of spikes per panicle; LP = length of the panicle; LBSR = length of the basal spike rachis; NSpP = number of spikelets per panicle; %SC = percentage of spikelets with caryopse

populations (Belgrano, La Plata, Magdalena and Monte) showed no relationship with geographic origin.

Bands 12 (1050 bp) and 40 (600 bp) included the variation in the first coordinate. Along this coordinate Pereyra Iraola is grouping. Second coordinate grouping Brandsen and Ranchos. Band 19 (750 bp) explained the variation in this coordinate. Finally, third coordinate grouped San

Table 9 Summary of the results of the amplification reactions performed by means of the primers assayed

Total number of primers	29
Number of polymorphic primers	10
Percentage of polymorphic primers	65.5%
Total number of polymorphic bands identified	60
Average number of polymorphic bands per polymorphic primer	6
Size average of polymorphic primer	730 bp
Size range of amplified products	250–1700 bp

Table 10 Size of polymorphic bands generated with 10 RAPD primers

Primer	Band	Size	Primer	Band	Size
A 08	1	500 bp	OPG 03	32	320 bp
	2	540 bp		33	420 bp
	3	550 bp		34	550 bp
	4	750 bp		35	650 bp
	5	1100 bp		36	700 bp
OPB 15	6	545 bp		37	950 bp
	7	575 bp	OPG 05	38	460 bp
	8	700 bp		39	480 bp
	9	770 bp		40	600 bp
	10	900 bp		41	650 bp
	11	950 bp		42	750 bp
	12	1050 bp	OPJ 13	43	480 bp
	13	1150 bp		44	490 bp
OPE 09	14	340 bp		45	570 bp
	15	400 bp		46	620 bp
	16	440 bp		47	850 bp
	17	670 bp		48	1100 bp
	18	725 bp		49	1300 bp
	19	750 bp	OPR 04	50	485 bp
OPE 18	20	250 bp		51	540 bp
	21	345 bp		52	575 bp
	22	550 bp		53	690 bp
	23	900 bp		54	1250 bp
	24	1000 bp		55	1700 bp
	25	1100 bp	P 124	56	400 bp
	26	1200 bp		57	430 bp
	27	1350 bp		58	575 bp
OPF 10	28	270 bp		59	1250 bp
	29	460 bp		60	1350 bp
	30	625 bp			
	31	1650 bp			

Vicente. Here the variation is explained by band 46 (620 bp) (data not shown).

Correlation analysis

The correspondence between morphological traits and RAPD markers was evaluated by means of a Mantel correlation test. The Mantel correlation (r) was 0.02463 and it was not significant, indicating that morphological and molecular markers were unrelated.

Discussion

Natural populations of *P. dilatatum* in the area sampled are mixtures of two biotypes, each with a distinct breeding system. It results in a complex situation where most plants are apomictic ($5x$) and others ($4x$) show sexual reproduction.

Efficient genetic characterization depends on levels of variation and in populations that are phenotypically similar unequivocal identification may therefore be complicated. However, high levels of variation within a population can also complicate the analysis (Caetano-Anollés 1998) and different methods and statistical techniques should thus be used to analyze genetic diversity.

Cluster analysis involves techniques that grouping the individuals associated by its similarity level. The grouping pattern of population based on morphological data did not reflect geographical origin. Instead this analysis grouped individuals of the different populations by ploidy level. Two groups were generated; one included pentaploid individuals and the other tetraploid individuals. Garita et al. (1994) reported similar results and suggested that, apart from the anther color and chromosome number, the characters that differentiate biotypes were: glumes pubescence, pedicel length, tiller length, pseudo-stem width, flowering date, pod color and rachilla fragility. Characters LP, LBSR, NRT and NSP allowed us to distinguish between tetraploid and pentaploid biotype (Table 8). Tetraploid and pentaploid individuals can therefore be considered as separate entities with own characteristics (Fig. 2). All vegetative characters were greater in the pentaploid biotype than in the tetraploid biotype and the same was true for all reproductive characters, except %SC (Table 4).

Even though the mean of reproductive characters were generally greater in the pentaploid biotype, %SC, the most important reproductive character because it indicates the contribution to the next generation, was higher in the tetraploid biotype, suggesting that the tetraploid biotype has a higher fitness. The higher value of NSP in the pentaploid biotype was compensated by a higher %SC in the tetraploid biotype. Production of spikelets with caryopse is 1.5- fold higher in the tetraploid biotype; tetraploid individuals produced 4.63% of spiklets with caryopse while pentaploid individuals produced 3.14%. Germination percentage showed similar differences between the two biotypes (data not shown). The percentage of germination was 25.5% for the

tetraploid biotype and 16.9% for the pentaploid biotype. The correlation between %SC and germination percentage was statistically significant ($P = 0.05$) and %SC can therefore be used as a predictor of seed fertility. Variation in %SC, was also lower in the tetraploid biotype compared to the pentaploid biotype.

Skroch et al. (1992) have found that the SM coefficient is an appropriate estimate of relatedness, assuming that among closely related plant materials, both the presence (1, 1) and absence (0, 0) comparisons of an amplified product in two or more genotypes result from the same genetic changes. Thus both kinds of comparisons provide nearly the same information. The grouping pattern of population based on molecular markers reflected geographical origin and did not reflect ploidy level of the individuals from different populations (Fig. 2). The pattern grouping was generated by differents primers. These results suggest that the populations of Brandsen, Pereyra Iraola, Ranchos and San Vicente, have unique genotypes which makes it possible to identify the geographic place of origin. On the other hand, the populations of Belgrano, La Plata, Magdalena and Monte showed very low levels of differences between them and all individuals clustered in a single, large group. This suggests that these four populations share some genotypes, thereby explaining the high interpopulation similarity. Also, gene flow between pentaploid and tetraploid individuals is possible because both coexist and hybrids can be formed between the two biotypes (Bennett et al. 1969). Bennett et al. (1969) got individuals $2n = 45$ in a cross between both biotypes where the source of pollen was the pentaploid biotype. Chromosomal number in individuals F_2 varied from 40 to 45 and 20 bivalents and from 0 to 5 univalents during meiosis. These individual F_2 showed sexual reproduction and heterosis. The cross effectiveness was 0.04%. Reciprocal crosses produced individuals with $2n = 70$ and they were infertile. Also, there is no references to individuals with $2n = 70$ in natural populations composed by tetraploid and pentaploid biotypes in the literature.

Morphological markers did not allow us to discriminate individuals by their geographic origin, probably because these characters are under environmental influence. However, molecular markers might reveal environmental adaptations that are not phenotypically expressed.

Cullis (1999) suggested that plants can develop the ability to respond to environmental pressures. Most of these responses are physiological and involve temporal reactions. However, there are cases of variation at the genome level as a response to environmental pressures. These variations include transposon activation, somaclonal variation, or gene amplification. In an evolutionary context it is necessary to identify regions involved in this type of variation because it will help us to understand how genomic reorganizations can lead to phenotypic variation. In addition, Cullis (1999) suggested that inbreeding species may house little genetic variation if they have been growing in a favorable environment for long time and this can likely be disastrous, unless a mechanism by which variation can be re-introduced or maintained is present. This reasoning can easily be extended to apomictic species as well.

RAPD markers are located at random in the genome. There is no evidence that these markers are located in specific regions (Ferreira and Grattapaglia 1996). In this way is possible to find them in coding or non-coding regions. Bassi (1999) suggested that non-codifying regions can be involved in gene regulation and DNA repetitive regions can modulate between environment and gene expression. In this way, our results might be showing adaptive modifications. Such modifications might generate the differentiation of the populations and the existence of unique genotypes in Brandsen, Pereyra Iraola, Ranchos and San Vicente. Even though the ecological characteristics of these places are very similar, the modifications of the genome might have been generated as the genotypes have adapted to each site, and the genomic structure might have been conserved despite although the fact those environmental pressures are no longer present.

Given the suggestions about the origin of *P. dilatatum* (Espinoza and Quarín 2000, Casa 1995) our results could indicate that different genomes were involved in the origin of the individuals belonging to the different populations analyzed. The *I* genome is present in different species of group Quadrifaria. *P. quadrifarium*

belongs at this group and this species is a component of the natural pastures (Josifovich et al. 1982) where samples were collected for this study. However, this alternative for explain the origin of both biotypes must be analyzed in detail.

The fact that there was no correspondence between the results obtained from molecular and morphological data suggests that the different types of markers probably are located in different regions of the genome. It could also be the consequence of natural selection on morphology that was not detected by molecular markers because of RAPD markers represents neutral variation.

Moreover, since RAPD did not discriminate between biotypes with sexual or apomictic reproduction, they are probably not associated with mating system.

The results show that polygenic traits such as LP, LBSR, NRT and NSP can be used to discriminate between biotypes whereas molecular markers such as bands 12, 40, 19 and 46 can be used to discriminate between populations, probably because they detect neutral variation.

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