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Genetic characterization of *Paspalum notatum* accessions by AFLP markers

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Abstract. *Paspalum notatum* Flügge is a warm-season forage grass with sexual diploid and apomictic tetraploid races. Genetic improvement was achieved in out-breeding diploids. The acquisition of artificial sexual tetraploids has raised the possibility of performing crosses and plant improvement at the tetraploid level. The objective of our study was to obtain a genetic and cytoembryological characterization of a germplasm collection of *P. notatum*, including 31 accessions from seven countries of America and 11 experimentally obtained genotypes. Morphology of mature gametophytes was observed to assess the mode of reproduction of the accessions. A total of 1342 AFLP fragments were generated across the 42 genotypes and from two reference taxa: *P. urvillei* and *P. procurrens*. AFLP data were converted into a binary matrix and similarity relationships were established. The genetic distance among all the accessions showed a maximum value of 0.36. In addition, eleven AFLP fragments were observed exclusively in apomictic plants, which could be linked to genomic regions implicated in the control of apospory.

Key words: AFLP analysis, apomixis, genetic diversity, *Paspalum notatum*, reproductive behaviour.

Introduction

Paspalum notatum Flügge (bahiagrass) was considered an excellent native forage grass long before it became a cultivated species. Natural populations are widely distributed in the New World from Mexico to Argentina and throughout the West Indies. The most common form of this species is the tetraploid ($2n = 4x = 40$), apomictic, pseudogamous, and self-compatible cytotype (Burton 1948, Bashaw et al. 1970). The wild diploid ($2n = 2x = 20$), self-incompatible sexual biotype inhabits a very limited area in South America (Burton 1946, 1955, 1967; Daurelio et al. 2004). Occasionally, triploid and pentaploid apomictic genotypes have been collected from natural populations (Quarin et al. 1989, Tischler and Burson 1995).

In the last century, diploid *P. notatum* was accidentally introduced into the United States, probably as a consequence of cattle transport (Burton 1967). The adventitious material was then improved by conventional breeding and brought into cultivation as Pensacola bahiagrass (Burton 1967). Currently, Pensacola

bahiagrass is one of the major forage grasses grown in the southern parts of the United States. By contrast, since apomixis prevents crossing, the improvement of tetraploid races has been historically limited to the identification and selection of superior introductions. Hanson (1972) recognized five selected tetraploid types growing in USA: Common, Argentine, Paraguay, Paraguay 22, and Wilmington. Another example of a tetraploid genotype that was selected for forage purposes is the cultivar 'Competidor' that was released in Australia in 1987 (Wilson 1987). Crossing is not possible between two obligate apomictic tetraploid races; hybridization only is achievable when a sexual tetraploid is available. Breeding apomictic tetraploid races through hybridization programs started with the acquisition of sexual tetraploid plants by colchicine treatment of sexual diploid Pensacola bahiagrass seeds (Burton and Forbes 1960). Induced sexual tetraploids set seed reasonably well and easily hybridized with the obligate apomicts. However, these breeding programs were rapidly abandoned because the inheritance of apomixis was not clearly understood, most of the individuals in the F_2 were inferior to their apomictic parent, and attempts to develop a superior sexual tetraploid population were unsuccessful (Burton 1984). In addition, the scarcity of a large and well-characterized germplasm collection of tetraploid apomictic genotypes restricted the possibilities of developing adequate breeding programs.

The analyses of diversity among and within populations, and the genetic characterization of elite germplasm, are central requirements to successful breeding programs. While assessment of diversity is important to evaluate the genetic base to be exploited via heterosis (Melchinger 1999), genetic characterization allows the optimal use of this potential. Different types of markers have been used for germplasm characterization and genetic diversity estimations. Biochemical methods have been applied to the differentiation of cultivars or genotypes in several grasses (Lin et al. 1984, Dabo et al. 1990), but molecular methods based on DNA analysis are considered to be the most accurate (Nybom 1994, Zhang et al. 1999). Random amplified polymorphic DNA (RAPD) have been used for measuring genetic diversity in several grass species, i.e. *Tripsacum* (Li et al. 1999), *Poa pratensis* (Johnson et al. 2002), and *Paspalum* (M'Ribu and Hilu 1996, Liu et al. 1994). Amplified fragment length polymorphisms (AFLP) have also been used to differentiate *Cynodon* (Zhang et al. 1999) and *Poa* genotypes (Larson et al. 2001). However, comparative studies using different molecular marker techniques such as RFLP, AFLP, RAPD, and SSR (simple sequence repeat) have shown that AFLP is the most efficient method to estimate the genetic diversity because of its high reproducibility and multiplex ratio (Powell et al. 1996, Russell et al. 1997, Pejic et al. 1998).

The objectives of our research were to; (i) characterize the entire germplasm collection of *P. notatum* held at IBONE, Corrientes, Argentina, (ii) assess the genetic distance among different accessions by AFLP, and (iii) cytoembryologically determine the reproductive mode of these accessions. The set of plants analyzed here includes a significant number of wild genotypes collected throughout the Americas together with some experimentally acquired material. Approximately one half of the accessions are reproductively examined in this work whereas the rest has already been characterized in previous studies (Martínez et al. 2001, Quarin et al. 2001). The current availability of tetraploid sexual genotypes obtained by experimental means (Quarin et al. 2001, Quarin et al. 2003) together with the use of the information provided here will allow an optimal re-initiation of breeding programs of the tetraploid genotypes of the species.

Materials and methods

Plant material. Forty-two *P. notatum* genotypes, including 31 wild germplasm accessions and 11 plants experimentally produced at IBONE were used in this study. Wild accessions were collected

from natural populations located at seven different countries from Cuba to Argentina. Accessions of *P. procurrens* and *P. urvillei* were incorporated as reference taxa to provide a reference point for estimating genetic similarities within the *P. notatum* genotypes when generating the trees. The source, ploidy level, reproductive behaviour and identification code for each genotype are indicated in Table 1.

Embryological studies. Eighteen accessions of *P. notatum* were analyzed in order to characterize their reproductive behaviour. In *Paspalum*, the structure of the aposporous embryo sac is easily distinguishable from the typical composition of gametophytes of meiotic origin. Moreover, sexual and apomictic plants usually show variation in position, orientation and number of embryo sac per ovule. Meiotic embryo sacs (MES) were characterized by the presence of an egg-apparatus, a bi-nucleated central cell, and a mass of antipodal cells at the chalazal end. Aposporous embryo sacs (AES) were easily identified due to the lack of antipodal cells. Fifteen accessions belonged to tetraploid collections gathered from a vast geographic region, and the other three polyploid genotypes (Q4090, Q4130 and Q4131) were produced through 2n + n crosses, where the female parent was apomictic. Inflorescences were collected at anthesis and fixed for 24 h in FAA (18 parts 70% ethanol: 1 part 37% formaldehyde: 1 part glacial acetic acid) and then maintained in 70 % ethanol at 4° C. Pistils were dissected from the spikelets, clarified according the technique of Herr (1971) and observed with a differential interference contrast microscope (DIC).

AFLP studies. Genomic DNA was isolated from young leaves of an individual plant by using the protocol described by Dellaporta et al. (1983) with minor modifications (Ortiz et al. 1997). The AFLP procedure was undertaken following the manufacturer's instructions of the AFLP Analysis System I (Life Technologies, GIBCO BRL) with minor modifications. About 900 ng of genomic DNA were 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 further amplifications. Pre-amplification primers had one selective nucleotide. Pre-amplification products were diluted (1:10) with 1X TE buffer and used as templates for selective amplification. Fifty combinations of the *Eco*RI and *Mse*I AFLP primers supplied by the manufacturer (containing three selective nucleotides) were used for selective amplification. An UNO Biometra thermocycler was used for both pre-amplification and selective amplification. Reliability was assessed by the use of duplicates samples. 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. Five µl 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 BIO-RAD Sequi-Gen electrophoresis cell connected to a PowerPac/3000 power supply. Amplification products were visualized by using the Silver Staining-System from Promega, and digitized or recorded in APC film (Promega).

Data analysis. Markers were visually scored from the gels with the aid of a light box for presence (1) or absence (0) of bands and data 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 analyzed by using the Infostat computational pack (Infostat 2002) for calculating the similarity coefficient and group clustering. The Jaccard coefficient ($J = a/[a + b + c]$ was used to construct a dendrogram by the unweighted pair-group method with arithmetical averages (UPGMA), where *a* is the number of bands common to both individuals, *b* the number of bands present in the first individual and absent in the second and *c* the number of bands present in the second individual and absent in the first one.

Results

Reproductive behaviour. Cytoembryological analyses indicated that all accessions were either obligate or facultative apomicts (Table 2). In some ovules the normal meiotic sac developed together with one to several aposporous embryo sacs. A few ovules showed

Table 1. Identification, cytoembryology and origin of different accessions of the *Paspalum* species characterized by AFLP

Identification[a]	Species and Accessions	Chromosome number (2n)	Reproductive behaviour [b]	Origin (collection locality or source)
	P. notatum			
ARG-01	Oberá	40	Judged to be Apo†	Misiones, Oberá.
ARG-02	Q3686	30	Apo (1)	Corrientes, 18 km N of Sauce, Paso Mula.
ARG-03	Q3778	40	Apo (2)	Corrientes, Palmar Grande.
ARG-04	Q3838	40	Apo (2)	Corrientes, Riachuelo stream and Route 12.
ARG-05	Q3845	40	Apo (2)	Corrientes, 18 km N of Sauce, Paso Mula
ARG-06	Q3931	40	Apo (Urbani p.c.)	hybrid # 44 (Q3664 X Q3853).
ARG-07	Q4064	40	Apo (*)	Corrientes, Saladas.
ARG-08	Q4084	20	Sex (3)	Santa Fe, Cayastá.
ARG-09	Q4086	40	Apo (3)	colchicine induced autotetraploid from diploid Q4084.
ARG-10	Q4090	40	Apo (*)	2n + n hybrid from triploid Q3686 X diploid Q3658.
ARG-11	Q4130	50	Apo (*)	2n + n hybrid from tetraploid Q3838 X diploid Q4084 .
ARG-12	Q4131	60	Apo (*)	hexaploid obtained by pre-anthesis self-pollination of tetraploid Q3838
ARG-13	Q4175	20	Sex	Santa Fe, 15 km W of La Criolla.
ARG-14	Q4186	40	Apo (Urbani p.c.)	hybrid # 2 (Q3664 × Q3853).
ARG-15	Q4187	30	Sex/Male sterile (Martínez p.c.)	experimental origin, Q4084 and Q3686 are in its background
ARG-16	Q4205	40	Sex (4)	derived from self-pollination of Q3664
ARG-17	Q4210	40	Apo(*)	Catamarca, 10 km of Catamarca on the road to El Rodeo.
ARG-18	Q4261	40	Male sterile/judged to be Apo	Corrientes, Santa Ana, male-sterile plant.
ARG-19	Q4270	40	Apo(*)	Santa Fe, Rosario, Patio de la Madera.
ARG-20	U46	40	Apo(*)	Córdoba, Río Cuarto.
ARG-21	U47	40	Apo(*)	Corrientes, Parque Mitre, male-sterile plant.
ARG-22	U48	40	Apo(*)	Corrientes, Mercedes.
BOL-01	Q3776	40	Apo (2)	Villa Tunari, Chapare region.
BOL-02	ST2369	40	Apo(*)	Santa Cruz de la Sierra, 2 km S of Salinas.
BRA-01	IB229	40	Apo (2)	Itaquí, RS.
BRA-02	Q3844	40	Apo (2)	Lagoa Vermelha, RS.
BRA-03	Q4008	40	Apo(*)	Aparecida do Taboado, MS.
BRA-04	Q4010	40	Apo (2)	Tres Lagoas, MS.
BRA-05	Q4011	40	Apo(*)	Tres Lagoas, MS.

BRA-06	Q4012	40	Apo(*)	Tres Lagoas, MS.
BRA-07	Q4016	40	Apo (2)	18 km S of Dourados, MS.
BRA-08	Q4022	40	Apo(*)	30 km E of Ponta Pora, MS.
BRA-09	Q4023	40	Apo (2)	30 km E of Ponta Pora, MS.
BRA-10	Q4029	40	Apo(*)	Coronel Sapucaia, MS.
BRA-11	Q4117	40	Apo (2)	unknown locality, RS.
CUB-01	Q4181	40	Apo(*)	Indio Hatuey Experimental Station, volunteer.
MEX-01	Q3775	40	Apo (2)	Tamaulipas.
PAR-01	N160	40	Apo(*)	Amambay, 25 km N of Pedro Juan Caballero.
PER-01	SV2893	40	Apo(*)	Departament of Cajamarca, Cajabamba, El Huayo, 2100 msm.
USA-01	Q3658	20	Sex (5)	Line # 2, experimental origin, Tifton, GA.
USA-02	Q3664	40	Apo (6)	Derived from sexual induced 4x plant crossed by an apomictic white-stigma bahiagrass strain at Tifton, GA.
USA-03	Q4160	20	Sex (7)	Commercial variety Tifton 9.
	P. procurrens			
ARG-23	Q4060	20	Sex (8)	Salta, 10 km S of J.V.Gonzalez.
	P. urvillei			
BRA-12	Q4226	40	Sex	Restinga Seca, RS.

[a] ARG: Argentina; BOL: Bolivia; BRA: Brazil; CUB: Cuba; MEX: Mexico; PAR: Paraguay; PER: Peru; USA: Unites States of America.
[b] (1) Altamiranda 1983, (2) Martínez et al. 2001, (3) Quarin et al. 2001, (4) Quarin et al. 2003, (5) Quarin and Burson 1983, (6) Quarin et al. 1984, (7) Burton 1989, (8) Quarin 1993. (*) Investigated in this work (see Table 2). p.c.: personal communication. Both obligate and facultative apomictic genotypes were marked as apomicts in the table. Only fully sexual individuals were marked as sexual. † Dead before bloom.

Table 2. Embryo sac types of eighteen accessions of *Paspalum notatum* (**MES**: meiotic embryo sacs; **AES**: Aposporous embryo sacs; **Ab/ImES**: aborted or immature embryo sacs)

Accessions	Number of ovaries scored	Percentage of ovaries with			
		MES	AES	AES + MES	Ab/ImES
N160	62	8.1	50	37.1	4.8
Q4008	45	15.5	60	20	4.5
Q4011	54	3.7	96.3	0	0
Q4012	46	0	100	0	0
Q4022	43	9.3	69.7	2.3	18.6
Q4029	56	23.2	48.2	26.8	1.8
Q4064	51	7.8	68.6	9.8	13.7
Q4090	86	10.5	54.6	10.5	24.4
Q4130	22	0	86.4	9.1	4.5
Q4131	20	0	80	15	5
Q4181	51	9.8	72.5	13.7	3.9
Q4210	50	8	66	26	0
Q4270	51	2.0	94.1	0	3.9
ST2369	47	17	70.2	10.6	2.1
SV2893	41	4.9	87.8	7.3	0
U46	46	8.7	63	23.9	4.4
U47	47	10.6	23.4	53.2	12.8
U48	47	0	100	0	0

immature, underdeveloped or even absent embryo sacs. Accessions U48 and Q4012 showed only AES, and they were classified as obligate apomicts. However, the other accessions formed MES, AES, or MES plus one to several AES and were classified accordingly as

Table 3. Number of bands, degree of polymorphism and markers exclusive of apomictic plants revealed by each AFLP primer pair for the 44 genotypes analyzed for *Paspalum notatum*, *P. simplex*, *and P. urvillei*. The numbers in brackets refer to bands considered exclusively in the genotypes of *P. notatum*

Primer Combinations	Number of analysed bands	Number of conserved bands	Number of polymorphic bands	Polymorphism rate (%)	Number of markers exclusive of apomictic genotypes
E-AAC + M-CAG	60 (39)	0 (6)	60 (33)	100 (84.6)	**0**
E-AAG + M-CAA	86 (58)	6 (22)	80 (36)	93.0 (62.1)	**1**
E-ACA + M-CTG	123 (94)	3 (24)	120 (70)	97.5 (74.4)	**0**
E-ACG + M-CTC	103 (63)	2 (17)	101 (46)	98.1 (73.0)	**0**
E-ACG + M-CTT	100 (82)	1 (13)	99 (69)	99.0 (84.1)	**0**
E-ACT + M-CTG	93 (65)	3 (17)	90 (48)	96.7 (73.8)	**1**
E-AGC + M-CTG	93 (64)	5 (13)	88 (51)	94.6 (79.7)	**1**
E-AGG + M-CTC	78 (50)	2 (10)	76 (40)	97.4 (80.0)	**1**
E-ACC + M-CAC	85 (60)	1 (15)	84 (45)	98.8 (91.6)	**1**
E-ACC + M-CAG	107 (77)	2 (22)	105 (55)	98.1 (71.4)	**0**
E-AAG + M-CTG	136 (93)	4 (28)	132 (65)	97.1 (69.9)	**1**
E-ACA + M-CAG	100 (67)	2 (12)	98 (55)	98.0 (82.1)	**4**
E-AGG + M-CAA	74 (52)	0 (14)	74 (38)	100 (73.1)	**0**
E-ACT + M-CAT	104 (67)	5 (20)	99 (47)	95.2 (70.1)	**2**
Total	**1342 (931)**	**36 (233)**	**1306 (698)**	**97.3 (74.9)**	**11**

facultative apomicts. The percentage of ovaries showing only MES among the different accessions varied from 0 to 23.2 %. The maximum potential for sexuality reached 63.8 % in U47 when counting the ovules with MES and those with AES + MES.

AFLP analysis. A total of 50 primer combinations from the 64 available in the manufacturer's kit were screened on the accession Q4117. Out of the total number of oligonucleotide pairs analyzed, only one sample (2 %) did not produce amplification products. Fourteen primer combinations were selected because they provided clear and scorable banding patterns suitable for determining genetic diversity among the different genotypes of *P. notatum*. Fragments from 50 to 800 bp were used for the analysis. A total of 1342 DNA fragments were generated across the 42 genotypes of *P. notatum*, a *P. urvillei* genotype, and a *P. procurrens* genotype. From the total bands observed 1306 (97.3 %) were polymorphic. However, only 931 fragments were scorable in the *P. notatum* genotypes (42 accessions), out of which 698 (74.9 %) were polymorphic. The number of bands detected by each primer combination was on average 96, and ranged from 60 (*Eco*RI-AAC/*Mse*I-CAG) to 136 (*Eco*RI-AAG/*Mse*I-CTG). Eleven DNA fragments were detected only in

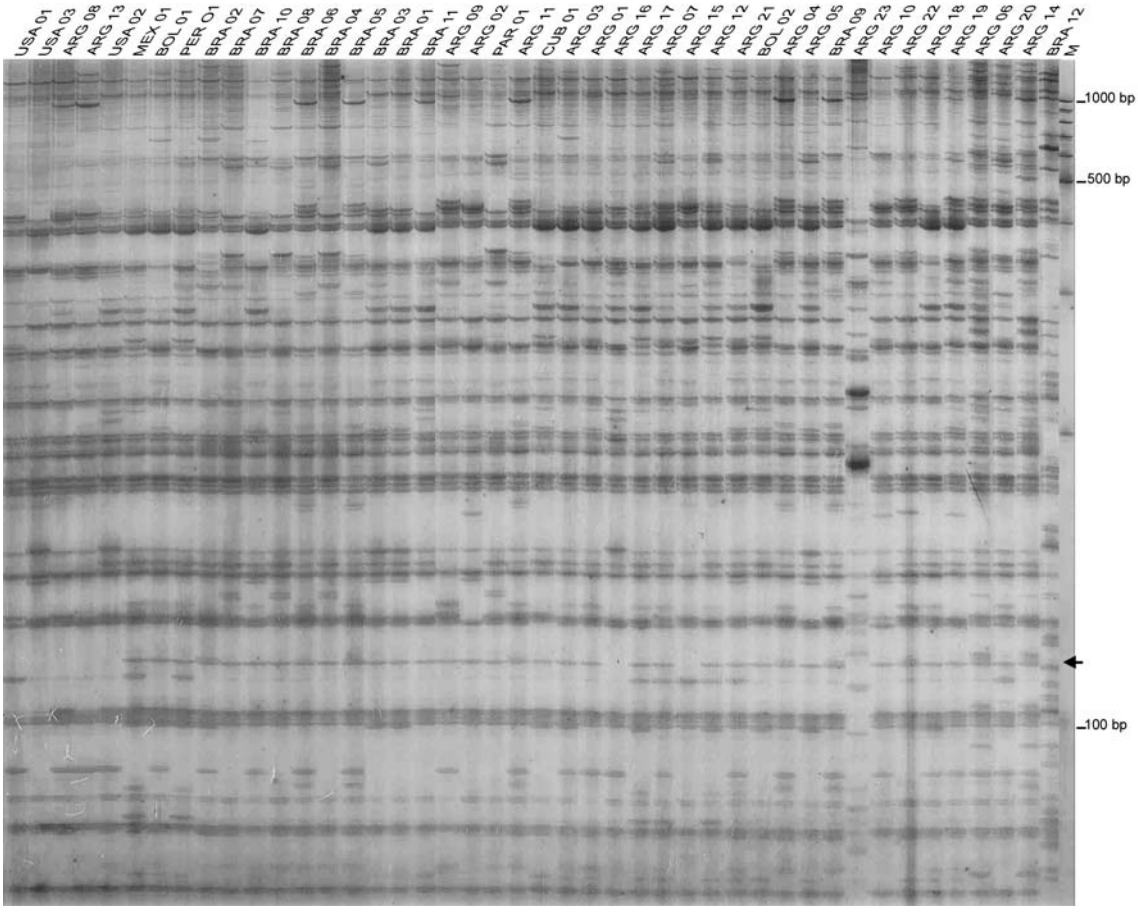

Fig. 1. Silver stained polyacrylamide gel visualizing amplifications patterns for the forty-two *P. notatum* genotypes and two reference taxa (corresponding to *P. urvillei* and *P. procurrens*) using *Eco*RI-AAG/*Mse*I-CTG primer combinations. M: Molecular weight marker. Arrow indicates a band present only in the apomictic genotypes

apomictic plants and were absent in all 2x and 4x sexual genotypes of *P. notatum*. The number of bands and the degree of polymorphism revealed by each primer combination are shown in Table 3. A representative example of the amplification products obtained with the *Paspalum* genotypes using the primer combinations *Eco*RI-AAG/*Mse*I-CTG is shown in Fig. 1.

A binary matrix was constructed with the data obtained from 1342 AFLP fragments and used to generate the genetic similarity esti-

Fig. 2. A phenogram obtained from a UPGMA based on the genetic distance values among the 44 *Paspalum* accessions studied. Apo: apomictic genotypes; Sex: sexual genotypes. Asterisk indicated apomictic genotypes grouped with sexual plant

mates. Coefficients of dissimilarity for the 44 accessions ranged from 0.01 to 0.85. UPGMA analysis allowed the differentiation of all genotypes analyzed in this study (Fig. 2). The phenogram showed a phenetic correlation of 0.953, indicating that there was little distortion between the phenogram and the similarity matrix. The dendrogram showed a minor and a major cluster. The minor cluster included the *P. urvillei* and *P. procurrens* species corresponding to the Dilatata group and the subgenus *Anachyris* of *Paspalum*, respectively. These species were included in the analysis as reference taxa and were well separated from the *P. notatum* genotypes. *P. urvillei* and *P. procurrens* showed a sharp dissimilarity (83%) both between them and compared to the whole group of *P. notatum* accessions. As expected, the reference taxa are obviously much more distant (at least one of them) than the targeted species in study.

The major cluster comprised all the genotypes of *P. notatum,* which is the most important species of the group Notata, an unofficial taxonomic category (Chase 1929) that actually includes a dozen species of *Paspalum*. Despite the vast geographic sources and the variety of environments of origin, all investigated accessions of *P. notatum* showed a remarkable degree of similarity. The genetic distance among all the accessions showed a maximum value of 0.36 (accessions ARG-06 and ARG-14 with respect to all other accessions), although in most accessions investigated in this work the dissimilarity ranged from 0.01 to 0.30 (Fig. 2).

In addition to these siblings, the fraction of the dendrogram corresponding to *P. notatum* showed two main clusters. One cluster contains most apomictic tetraploid genotypes, including the pentaploid ARG-11 and the hexaploid ARG-12. In this group, two interesting small assemblages could be distinguished in relation to morphological characteristics that distinguish them. One association involves ARG-04, ARG-11, BRA-02, BRA-05, BRA-06 and BRA-09, all of them bearing thin and short leaf blades, which are uncommon peculiarities for polyploid biotypes. In contrast, the other assemblage BRA-04, BRA-07, BRA-08, and PAR-01, all of them presenting characteristic wide and short leaf blades and widely divaricate, often arcuate racemes in mature inflorescences. This particular phenotype is frequently found among tetraploids in the tropical regions of Brazil and Paraguay.

The second important cluster within the *P. notatum* group contains mainly sexually reproducing individuals, with the exception of apomictic ARG-02, ARG-10 and USA-02. All sexual genotypes are diploid or tetraploid derived from sexual diploids. Interestingly, the apomictic genotype ARG-02 included in the group of sexual accessions is a natural triploid that was collected in a locality where sexual diploid biotypes may occasionally be found in the general distribution area of wild diploids, whereas ARG-10 is an apomictic tetraploid plant experimentally derived from apomictic 3x ARG-2. ARG-10 was obtained as a 2n + n hybrid by pollinating the 3x ARG-2 plant with pollen of the diploid plant USA-01. These two genotypes clustered in the same subgroup. The third genotype that fell into the group of sexual plants was USA-02, an apomictic 4x plant with a high degree of sexual reproduction (> 70%) derived from crosses between a sexual colchicine-induced 4x plant and an apomictic natural tetraploid.

Discussion

In this study we provide information regarding the reproductive mode of eighteen accessions of *P. notatum* collected from the wild. Most of these accessions are facultative apomicts with a varying degree of residual sexuality, while a few accessions appear to be obligate apomictic (only aposporous embryo sacs observed). These results are consistent with those previously published by our group (Martínez et al. 2001) in which all the 19 tetraploid collections of bahiagrass were facultative apomictic. In summary, all of the more than thirty tetraploid accessions of the species, collected in Brazil,

Argentina, Bolivia, Paraguay, Peru, Cuba, and Mexico are apomictic, and mainly facultative aposporous apomictic. No sexual tetraploid plants have ever been found across the wide distribution area of the species, from Mexico to central Argentina and Uruguay. However, sexual 4x plants could be experimentally obtained through colchicine-treatment of sexual 2x cytotypes. These induced sexual tetraploids generated fertile progenies when pollen of indigenous apomictic tetraploids was provided (Forbes and Burton 1961, Quarin et al. 2001).

Tetrasomic inheritance of a major dominant gene has been proposed as the genetic control of apospory in this species (Martínez et al. 2001), in agreement with the genetic control of this trait in other tropical grasses (Savidan 1981, Sherwood et al. 1994, do Valle et al. 1994). Since crossed sexual and apomictic genotypes in *P. notatum* segregate for reproductive mode in the F_1 generation, and self-pollinated sexual F_1 plants always produce sexual F_2, it is easy to understand why wild apomictic 4x plants are heterozygous for apospory. Moreover, most authors have proposed a simplex heterozygous condition (+ − −) for apomictic autotetraploid grasses e.g: Savidan (1981) for *Panicum maximum*, Sherwood et al. (1994) for *Cenchrus ciliaris*, do Valle et al. (1994) for *Brachiaria*, and Martínez et al. (2001) for *P. notatum*. The question that remains unanswered is why there is a lack of entirely sexual tetraploid plants in natural populations? Since there are facultative apomictic individuals heterozygous for apospory (simplex condition), sporadic events of sexual reproduction expected in facultative apomicts should segregate for absence of aposporous reproduction. However, totally sexual 4x individuals have never been found in wild populations of *P. notatum*, and our results confirm that entirely sexual 4x individuals do not exist among natural populations of apomictic bahiagrass, despite the observations of some degree of facultativeness in embryological studies. Experimental work through progeny tests of facultative apomictic genotypes has been started in an attempt to recover 100% sexual plants of 4x *P. notatum*. Positive results would indicate that there is a strong natural selection against sexual 4x plants in natural populations. The degree of sexuality of the facultative apomictic individuals is also uncertain. Usually, the degree of sexuality is estimated by embryological approaches, where the percentage of ovules bearing embryo sacs with the typical structure of meiotic megagametophytes indicates the degree of potential sexuality. Whether or not these sacs will ultimately produce an embryo through fertilization (n + n) should be determined by progeny tests.

AFLP analysis allowed the differentiation and assessment of the genetic variability among 42 genotypes of *P. notatum*. It was also possible to determine the genetic distance between the *P. notatum* genotypes and two different species, *P. urvillei* and *P. procurrens* that belong to the Dilatata group and the subgenus *Anachyris*, respectively. These two species grouped with the *P. notatum* accessions at only 15% of similarity. However, the variability among the *P. notatum* genotypes ranged from 0.01 to 0.36, indicating a relatively small genetic diversity. These values are in agreement with those observed in *P. dilatatum* accessions where the genotypes showed a degree of similarity higher than 76% despite of their different ploidy levels (Casa et al. 2002). In contrast, when RAPD markers were applied to determine variability in accessions of *P. scrobiculatum* (kodo millet) similarity values varied from 21% to 75% (M'Ribu and Hilu 1996). These contrasting results may be because apomixis is the predominant condition in *P. notatum* and in *P. dilatatum*, while *P. scrobiculatum* is sexual, and is one of the few species of *Paspalum* native to the Old World tropics. *Paspalum scrobiculatum* was domesticated in India some 3000 years ago (de Wet et al. 1983) and harvested as a wild cereal. Sexual reproduction and coexistence of wild, weed, and cultivated types of *P. scrobiculatum* in its range of present-day cultivation account for its high degree of genetic diversity.

The low degree of genetic variability observed for *P. notatum* might be explained by the fact that the species operates as an agamic complex (Daurelio et al. 2004). Sexual diploid strains grow only in a limited area in Argentina that is considered the centre of origin of the species (Burton 1967). The wide distribution of tetraploid genotypes in South and Central America and the Caribbean Islands is thought to be a consequence of the spread of successful individuals probably originated in polyploidization events associated with the coexistence of diploid and tetraploid genotypes in the area of origin (Daurelio et al. 2004). However, our study showed that little differences exist between genotypes collected from distant locations, e.g. BOL01 and ARG03. In addition, the occurrence of polyploidy could generate more diversity than isolation by large geographical distances. For example, ARG12 is a 6x plant derived by sexual polyploidization (2n + n) from self-pollination of tetraploid ARG04. Recurrent polymorphisms were observed among these highly related plants, while the 4x genotypes BOL02 and CUB01 gathered from very distant sources are almost genetically identical (see Fig. 2). Extensive genome modification induced by a change of ploidy was observed using RAPD and AFLP analysis during the generation of newly synthetized autotetraploids in *Paspalum* (Martelotto, personal communication). Our data suggest that there is no agreement between genetic distance and geographic distance among the accessions of *P. notatum*. The small genetic variation among tetraploid *P. notatum* accessions from ecologically very different areas and of distant origins suggests that apomixis and polyploidy buffered the genetic status of the species providing an exceptional fitness for a wide range of environments (Daurelio et al. 2004).

We were able to identify 11 bands that were present only in the apomictic genotypes and absent in sexual genotypes. These bands associated with apomictic genotypes could be linked to genomic regions implicated in the control of apospory (Martínez et al. 2003, Stein et al. 2004). These markers have a great incidence in the separation of most apomictics from sexual genotypes in the dendrogram (Fig. 2). Further research are underway to confirm the linkage of these DNA sequences with apomictic genotypes of a segregating population obtained from sexual x apomictic crosses of tetraploid *P. notatum*.

The assessment of the genetic distance among the different genotypes of *P. notatum* reported here will allow the selection of the most adequate accessions to perform optimal crosses for apomixis genetic studies and for the breeding program currently held at IBONE. Finding molecular markers linked to the gene/s governing apomixis requires mapping of segregating progenies that are usually obtained by crossing completely sexual mother plants X full apomictic pollen donors. In order to easily detect polymorphic markers linked to the character, these parental plants should ideally be as polymorphic as possible. Availability of the dendrogram generated here will facilitate choosing plants with different mode of reproduction that are genetically distant and can be optimally used as parents during generation of progenies segregating for apomixis. On the other hand, the studies aimed at the identification of transcripts differentially expressed in apomictic and sexual plants require the comparison of near-isogenic material of contrasting reproductive mode. Again we can use the dendrogram to locate accessions showing different reproductive mode but close genetic structure to optimally perform comparisons. The information reported here together with the sexual crossing of experimental sexual tetraploid genotypes will also allow the rapid generation of highly heterotic new tetraploid individuals. Therefore, a tetraploid conventional crossing and selection-based breeding program for this very promising forage grass will be finally established.

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