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Genetic diversity in sexual diploid and apomictic tetraploid populations of *Paspalum notatum* situated in sympatry or allopatry

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Abstract. *Paspalum notatum* is a subtropical grass widely distributed in the temperate areas of America. Diploids are sexual while polyploids give rise to clonal seeds through aposporous apomixis. RAPD markers were used to analyze the genetic structure of three natural populations: i) diploids reproducing sexually (R2X); ii) sympatric apomictic tetraploids collected in the vicinity of the diploids (R4X); iii) allopatric apomictic tetraploids growing in isolation (C4X). The apomictic reproduction rate was evaluated by the use of molecular markers in progeny tests, while chromosome-counting allowed the verification of ploidy levels. Data revealed that the R4X group presented a variation considerably higher than that observed for C4X. Jaccard's coefficients were used to produce a cluster diagram using the UPGMA method. All but one tetraploid genotypes grouped together and were associated to diploid genotype A21. The possibility of occasional generation of novel tetraploid clones from the interaction between tetraploid and diploid individuals is discussed.

Key words: Agamic complex, apomixis, *Paspalum notatum*, ploidy levels, variability.

Introduction

Paspalum notatum Flügge is a perennial rhizomatous forage grass recognized as one of the major constituents of the native grasslands in the New World from Central Eastern Mexico to Argentina and throughout the West Indies (Chase 1929). From the first report of apomictic reproduction in this species (Burton 1948) it has been generally accepted that most Bahiagrass biotypes are autotetraploid ($2n = 4x = 40$) and reproduce through pseudogamous aposporous apomixis (Forbes and Burton 1961, Burton 1948). A sexual diploid race ($2n = 2x = 20$) native to North Eastern and Central Eastern Argentina was also described. This diploid form reproduces sexually by regular meiosis and is highly self-incompatible (Burton 1955). It was accidentally introduced to Pensacola, Florida, USA, and then brought into cultivation as a pasture and turf grass in the Gulf States (Burton 1967). All tetraploid biotypes are usually considered to be the typical form of the species *P. notatum* in

botanical terms. Otherwise, all diploid biotypes show distinctive morphological characteristics that fall into the botanical variety *P. notatum* var. *saurae* Parodi. Triploid ($2n = 3x = 30$) and even pentaploid ($2n = 5x = 50$) individuals have also been occasionally collected from the natural populations (Gould 1966, Quarin et al. 1989, Tischler and Burson 1995). The triploid studied by Quarin et al. (1989) has the morphological characteristics of *P. notatum* var. *saurae* and is visually indistinguishable from diploid strains.

Apomictic populations are regarded to be genetically uniform as a consequence of their clonal reproduction via seeds. However, a considerable variation rate has been observed in natural populations of both facultative and obligate apomictic polyploids. A model for the evolution of agamic complexes involving diploid-tetraploid-haploid cycles was proposed, based on the studies performed in the complexes *Capillipedium-Dichanthium-Bothriochloa* (De Wet 1968) and *Panicum maximum* (Savidan and Pernès 1982). According to this model, the occurrence of gene flow between sexual diploids and apomictic polyploids might allow the generation of at least some variability at the tetraploid level. Therefore, a greater diversity would be expected in areas where the tetraploids are sympatric with sexual races than in those where agamic populations grow in isolation.

Several studies analyzed the rate of variability of agamic populations that were in contact with sexual races. Considerable variability (sometimes comparable to that of the sexual populations) was observed in apomictic polyploids of the *Antennaria rosea* complex (Bayer 1990), *Boehmeria spicata* (Yahara 1990), *Taraxacum* sect. *Ruderalia* (Menken et al. 1995), and *Amelanchier* (Campbell et al. 1999). However, results obtained from the comparison of variation in sympatric and allopatric populations from the *Ranunculus auricomus* agamic complex (Hörandl et al. 2001) showed that a tetraploid population sympatric to sexual diploids presented depressed variation rates similar to those of the allopatric ones or even lower. The objective of this work was the evaluation of variability in apomictic tetraploid populations of *Paspalum notatum* growing in sympatry and allopatry with sexual diploids. The existence of both allo- and sympatric natural populations of diploid and tetraploid *Paspalum notatum* in north-eastern Argentina provided the opportunity to carry out a comparative study of genetic and genotypic variation.

Materials and methods

Plant material. The sympatric populations R2X and R4X grow at the margins of the Riachuelo stream, an affluent of the Paraná river, 15 km south-east from the city of Corrientes, Argentina. The R2X population predominates on the inundation bed very close to the stream margins, while R4X is mostly present on an alluvial terrace located a few meters above. Both patches of soil are connected by a gentle slope, where cytotypes from both populations coexist in close association. Approximately 40 pieces of rhizomes from population R2X were collected over nearly 1000 m^2. Cuttings 5 cm long were taken at least 5 m apart from each other to avoid sampling of a single individual. Similarly, about 40 rhizome samples were obtained from the contiguous R4X population. Both collections included a few individuals from the sloped area where 2x and 4x populations overlapped. Ploidy discrimination was initially based on the phenotypic characteristics of the diploid and tetraploid cytotypes, mainly leaf width and spikelet size. Rhizomes were transplanted in pots and cultivated in a greenhouse. The allopatric population C4X was located 11.5 km south-east from the city of Castelli, Provincia del Chaco, Argentina. This group of individuals is geographically isolated and distant more than 200 km from any diploid sexual population of the species. Rhizomes were collected from the borders of an old local road in a total area of about 1200 m^2. All 40 plants sampled showed the typical phenotype of the tetraploid races of *P. notatum*. Plants were grown and maintained in a greenhouse as indicated above. Out of the 40 original genotypes collected, only the group of plants that grew vigorously in the greenhouse (27 for R2X, 30 for R4X and 30 for C4X) were selected to perform the cytogenetic and genetic studies. A progeny test survey was carried

out to check the rate of apomictic reproduction on four different progenies, obtained by open pollination of individuals R4X9, R4X12, C4X5 and C4X21. Progenies consisted of 24, 22, 18 and 18 individuals, respectively. The distribution of the populations used in this study is shown in Fig. 1.

Chromosome counting. Chromosome numbers were determined in root tips that were pretreated with an α-bromonaphthalene aqueous-saturated solution for 2 h, hydrolyzed in hydrochloric acid (1 N) for 10 min at 60°, stained with basic fucsin and squashed in a drop of 2% aceto-orcein. Chromosomes were observed with a transmission light microscopy.

RAPD analysis. Genomic DNA was extracted from 4 g of fresh leaves by using the method of Dellaporta (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

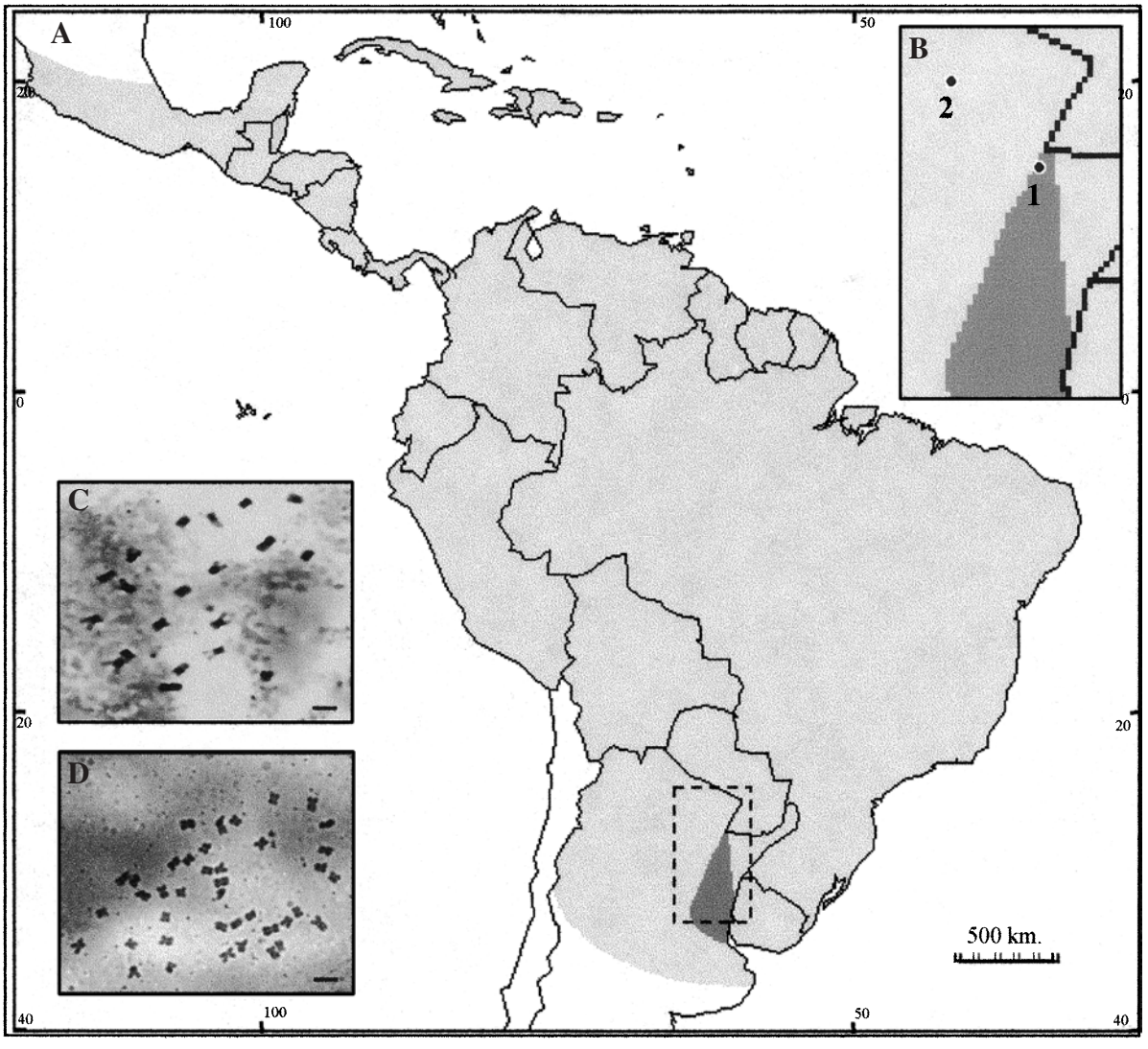

Fig. 1. Natural distribution of *Paspalum notatum* cytotypes in Central and South America. Distribution of diploids is shown in dark grey and tetraploids in light grey (panel **A**). A close-up showing the site of collection of populations R2X and R4X (1) and population C4X (2) is displayed at the top right (panel **B**). Microphotographs of mitotic metaphase chromosomes for a diploid (2n = 20) and a tetraploid (2n = 40) plant are shown in panels **C** and **D** respectively (Bar = 5 μm)

integrity and absence of RNA contamination. Each RAPD amplification was performed in a reaction volume of 25 μl containing 30 ng primer, 20 ng genomic DNA, 1X Taq polimerase reaction buffer (Promega), 15 μM each dNTPs, 1.5 mM magnesium chloride and 1 U of Taq polymerase (Promega). Decamers used belonged to the University of British Columbia series 5 (401–450) and 8 (701–751). Amplifications were carried out in a Biometra UNO-Thermoblock programmed as follows: an initial denaturation at 93°C for 1 min, 45 cycles of 1 min at 92°C, 1 min at 36°C and 1.5 min at 71°C and a final extension step of 5 min at 72°C. For the intrapopulation diversity studies, an initial amplification using primers BC420 and BC431 proved the quality of the 87 DNA samples used in the assays. Negative controls were included to exclude contamination of the reagents. Reliability and repeatability was assessed by running duplicate reactions. Amplification products were analyzed by electrophoresis in 2% agarose gels run at 4–5 V/cm for 2-3 h in TAE 1 X buffer and stained with ethidium bromide. Alternatively, amplification products were run in 5% polyacrylamide gels, electrophoresed in TBE 1X and silver-stained by using the Silver Staining System from Promega. Gel images were digitized or recorded in APC film (Promega) For comparison of analogous bands from different populations a molecular marker (Cien Marker, Promega) and an R2X control sample were electrophoresed alongside.

Data analysis. Data were converted into a binary matrix by using the Microsoft Excel program, where 1 indicated the presence of a given band, 0 its absence and a blank space a data missed by failure of amplification or the occurrence of unclear/poorly-defined bands. Matrices were analyzed for the determination of similarity coefficients between pairs of individuals and group clustering by using the InfoStat computational pack (Facultad de Ciencias Agropecuarias, National University of Cordoba, Argentina). In all cases the Jaccard's coefficient (J) was used ($J = a/[a + b + c]$ 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 one and c the number of bands present in the second individual and absent in the first one (Jaccard 1908). A group clustering analysis was performed through the UPGMA method. The cophenetic correlation was considered to be confident enough for values above 0.9 (Huff 2001).

Genetic variation was estimated through: a) the coefficient of variation ($CV = \sigma/\mu$, where σ is the standard deviation and μ is the average value of the Jaccard similarity indices) and b) the percentage of polymorphic markers within each population. Genotypic variation was evaluated by following the methods of Ellstrand and Roose (1987) and Eckert and Barret (1993) commonly used for clonal populations. Each multilocus genotype was defined from the global banding pattern considering all markers. An individual was regarded to be a distinct genotype when the corresponding amplification pattern presented at least one differential band with respect to the fingerprints produced by the rest of the individuals. The number of different multilocus genotypes in each population was called G. The proportion of distinct multilocus genotypes (''proportion distinguishable'') was calculated as G/N, where N is the total number of individuals in the population analyzed. The multilocus genotype diversity (D) was calculated as the index of Pielou (1969), $D = 1 - [n_i (n_i - 1)]/[N (N - 1)]$ where n_i is the number of the *i*th genotype observed (*i*: 1 to G). The multilocus genotypic evenness (E) was calculated as $E = [D - D_{min.}]/ [D_{max.} - D_{min.}]$, where $D_{min.} = [(G\text{-}1)(2N - G)]/ [N(N - 1)]$ and $D_{max.} = [N(G - 1)]/ [G(N - 1)]$. D and E values ranges from 0 (for an uniclonal population) to 1 (in a population where each individual has a different genotype).

Results

Progeny tests. An initial estimation of the rate of vestigial sexual reproduction in tetraploid genotypes from populations R4X and C4X was essential to determine the possible variability generated by occasional recombination. Progenies obtained by open pollination from four randomly-selected individuals from the R4X and C4X populations were analyzed by amplification with the RAPDs primers BC708 and BC726. Both oligonucleotides together generated around 50–70 markers in polyacrylamide gels, depending on the progeny. All the individuals in the different progenies from both populations presented a genetic pattern

identical to that of the mother plant, which confirmed that they had been generated from an apomictic reproductive event. Based on these results the rate of residual sexuality might be considered insignificant in these representative genotypes of populations R4X and C4X and therefore the plants can be regarded as obligate apomicts.

Chromosome counting. Individuals collected in the field were originally classified as diploid or tetraploid based on its phenotypic characteristics. Chromosome counting was performed for each individual plant. One plant of R2X population showed $2n = 4x = 40$ chromosomes and then was reconsidered as a member of the neighboring R4X population. Similarly, one plant from R4X was changed to population R2X because it showed $2n = 2x = 20$ chromosomes. An illustration of the chromosome counting preparations is showed in Fig. 1.

RAPD analysis of intrapopulation variation. Selection of informative oligonucleotides was done on two individuals from R2X population based only in the amplification of clear and reproducible bands. Out of the 101 total oligonucleotides assayed, 79 generated unambiguous amplification patterns, eight showed no amplification at all and 14 only faint or smeared bands. Out of the total number of primers that produced satisfactory amplification profiles, 25 were selected for the reactions to be reproduced, based on the abundance and quality of the bands. Finally, six decamers (BC429, BC445, BC732, BC745, BC431 and BC726) were assayed on two individuals from each population prior to be used on the totality of the samples. Amplifications generated 70 total markers, which were represented by clear reproducible bands in all tests. The total number of bands considered and the percentage of polymorphic bands for each primer within each population are indicated in Table 1. Examples of acrylamide gels showing the characteristic amplification patterns obtained are shown in Fig. 2.

Genotypic variation. The genotypic variability is related to the number of multilocus genotypes (defined by the banding pattern in all loci considered) that can be detected in a population (Ellstrand and Roose 1987). Different genotypic diversity coefficients commonly used for clonal population studies were calculated (Ellstrand and Roose 1987, Campbell 1999): the number of different multilocus genotypes (G), the "proportion distinguishable" (G/N), the multilocus genotypes diversity (D) and the genotypic evenness (E). Index values

Table 1. Score of markers obtained from the RAPD amplification of the three populations

Primer	Sequence	Gel	Population								
			R2X			R4X			C4X		
			TB	PB	% PB	TB	PB	% PB	TB	PB	% PB
BC429	AAACCTGGAC	Ag.	3	1	33.3	3	1	33.3	3	0	0.0
BC445	TAGCAGCTTG	Ag.	6	6	100	5	4	80.0	5	1	20.0
BC732	CACCCACCAC	Ag.	4	4	100	4	2	50.0	4	2	50.0
BC745	GGGAAGAGGG	Ag.	4	3	75.0	4	0	0.0	4	1	25.0
BC431	CTGCGGGTCA	Pol.	30	27	90.0	30	21	70.0	24	2	8.3
BC726	GGTGTGGGTG	Pol.	21	14	66.6	20	10	50.0	18	1	5.5
Total			68	55	78.6	66	38	54.3	58	7	10.0

Oligonucleotide sequences (5′–3′) and the type of gel used (Ag.: agarose gel, Pol.: polyacrylamide gel) are indicated. TB, PB and % PB indicate the total number of bands considered, the number of polymorphic bands and the percentage of polymorphic bands, respectively.

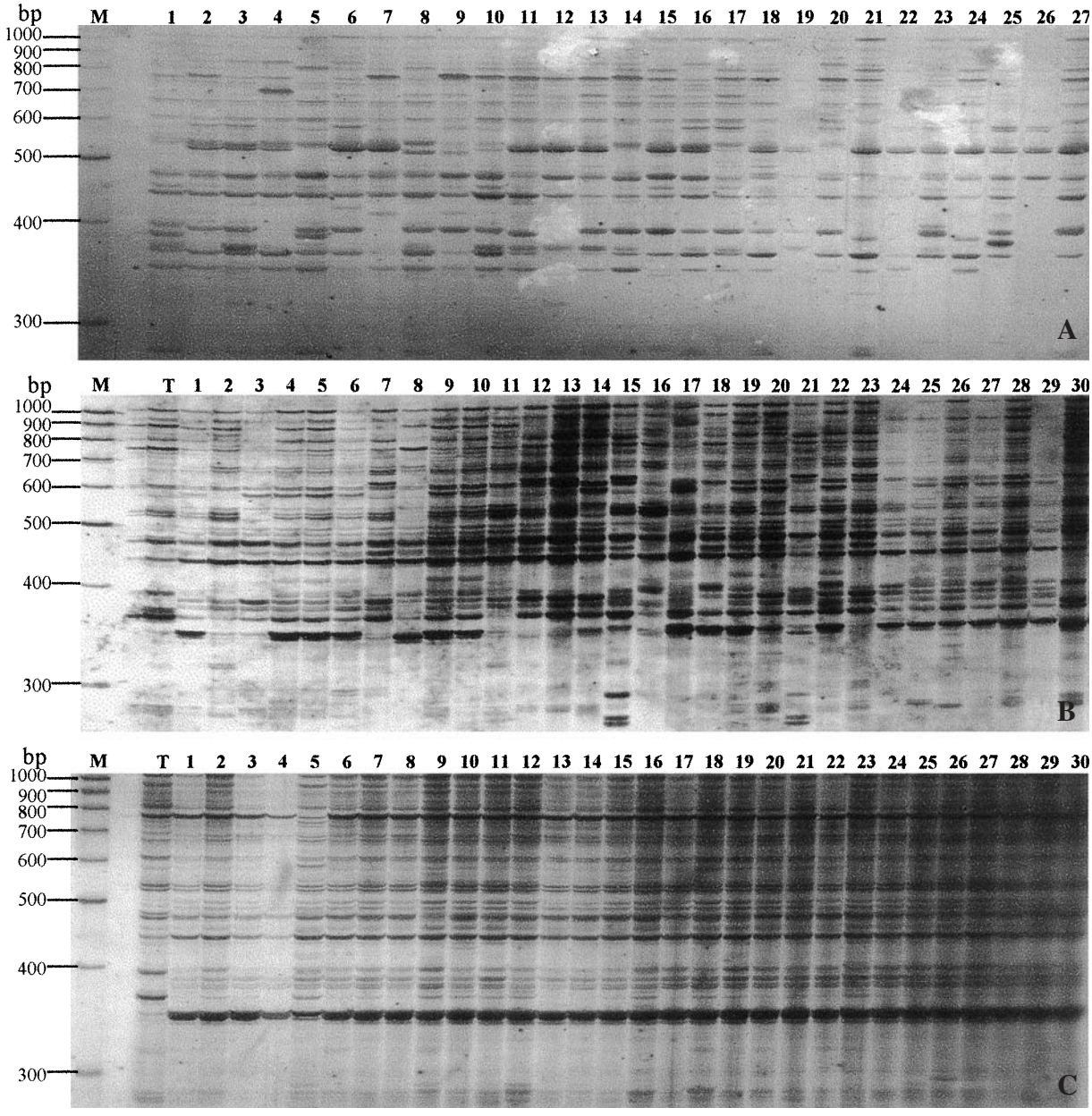

Fig. 2. RAPD amplification patterns for the three *P. notatum* populations. Bands were produced by amplifying genomic DNA with oligonucleotide BC431. Samples were electrophoresed in 5% polyacrylamide gels. Panels **A**, **B** and **C** display the fingerprints corresponding to individuals from populations R2X, R4X and C4X, respectively. M: molecular marker (Cien Marker, Promega). T: control sample (R2X18)

obtained are shown in Table 2. Genotypic variation resulted maximal for R2X with G/N, D and E values of 1 or close to 1, as it was expected from the reproductive characteristics of the population, which is composed by sexual plants allogamous by a self-incompatibility system. The sympatric R4X population showed values of genotypic diversity indices that can be regarded as high if considering its apomictic reproductive system, and many different genotypes could be discriminated. Opposedly, C4X presented a very low genotypic variation

Table 2. Estimates of genetic and genotypic variation for the three populations of *P. notatum*

	Index							
Population	N	G	G/N	D	E	μ	CV	%PA
R2X	27	27	1.00	1.00	1.00	0.63	11.9	23.5
R4X	30	27	0.90	0.99	0.46	0.81	9.7	3.0
C4X	30	5	0.17	0.45	0.34	0.99	1.8	0.0

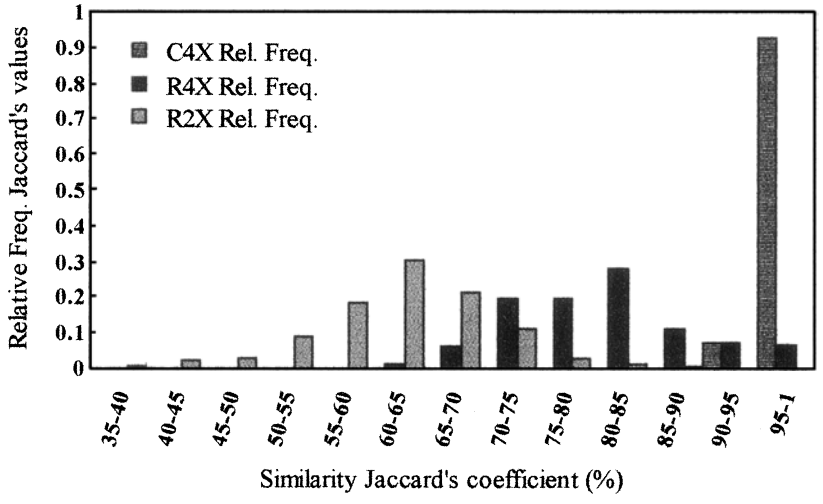

Fig. 3. Histograms of Jaccard's similarity coefficient for populations R2X, R4X and C4X. Relative frequencies of the similarity index are shown as a function of intervals of Jaccard's values

compared to the other two populations studied here and other clonal population analyzed in previous works (Campbell et al. 1999, Hörandl et al. 2001). Almost all C4X individuals were genetically identical, and the few ones that could be distiguished as unique showed a genetic constitution very similar to that of the main clone.

Genetic diversity. The mean value and the coefficient of variation (CV) of the Jaccard similarity indices for each pair of individuals were used to estimate the genetic diversity within each population. The CV can be considered a global indicator of the genetic variability independently of the number of genotypes studied (Nieto-Lopez et al. 2000). Genetic variability resulted maximal for R2X, intermediate for R4X and minimal for C4X (Table 2). Significant differences in genetic variation values can be observed between the apomictic populations R4X and C4X. On the other hand, the contrast between R2X and R4X population data is less evident for the CV value compared to the mean value because of the particular distribution pattern of the Jaccard indices (see Fig. 3). The percentage of polymorphic bands observed within each population (Table 1) is another genuine indicator of the rate of genetic variability showing again a higher variability in R2X with respect to the other two populations and a diversity relatively high in the R4X compared to C4X.

Clustering analysis. Data considering the presence or absence of bands were processed by multivariate analysis to obtain a clustering based on the genetic distance (1-Jaccard). Such study was performed by using the UPGMA method and allowed the construction of an unrooted tree showing clustering of individuals (Fig. 4). All R4X individuals except one (R4X30) appeared included in a single group. The only individual that escaped this condition was precisely the one that had been phenotypically misclassified as a diploid and reclassified as a tetraploid based on the cytogenetic studies. On the other hand, all C4X individuals

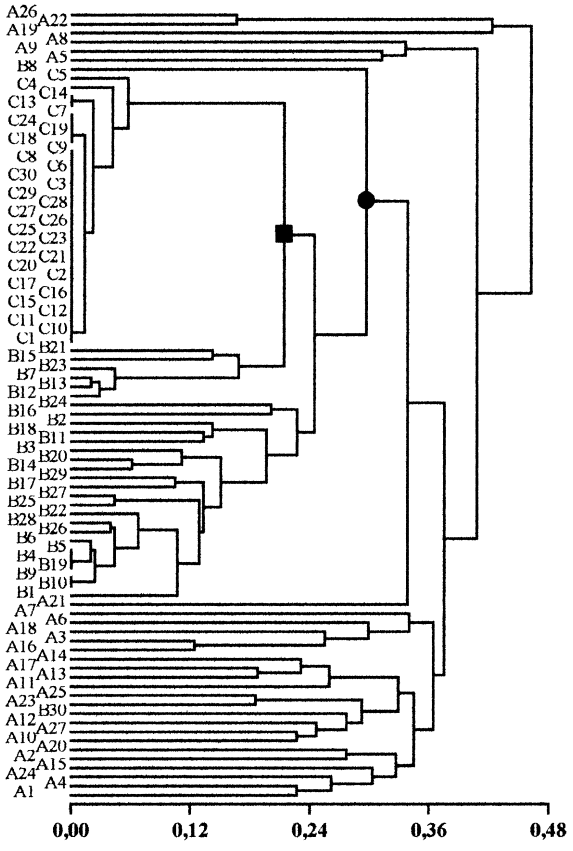

Fig. 4. Cluster analysis (UPGMA) obtained from the genetic distance values among the 87 *P. notatum* individuals studied. A: R2X individuals, B: R4X individuals, C: C4X individuals. The black box indicates the closer tetraploid common origin. The black oval marks the C4X population common origin with a group of individuals from R4X population. Genetic distances (1-Jaccard) are shown at the bottom

were grouped with the majority of the R4X tetraploids. Genetic distances within populations confirmed a higher genetic variability rate in R2X, intermediate in R4X and lower in C4X. Both tetraploid groups (R4X, C4X) appear to be more closely related that either is to R2X. However, the genetic distance among all the tetraploids (R4X, C4X) and most of the diploids is shorter than that among some of the diploids themselves (i.e. individuals A22, A26, A19, A8, A9). The diploid most closely related to the tetraploids is A21. The phenogram showed a phenetic correlation of 0.930, indicating that data used were substantial.

Discussion

The dominant view in the first half of the past century was that populations reproducing apomictically almost lacked genetic variation (reviewed in Asker and Jerling 1992). Gustafsson (1947) opened a new vision of the evolutive possibilities of apomicts by postulating that: a) many of them can retain at least some sexuality and b) somaclonal variation may occur from point mutations, autosegregation, somatic crossing-over or chromosome rearrangements. Therefore, if the last vestiges of sexuality were somehow eliminated from asexual populations, apomicts would become actual evolutionary dead ends for which the only possible way out from uniformity would be random mutation.

The chance of variability invading apomictic systems by the periodic formation of new genotypes from sexual individuals of lower ploidy level was first envisaged by De Wet (1968) and later by Savidan and Pernès (1982), who proposed a model where variability would be generated at the diploid level by usual sexual recombination and pumped up to the polyploid level through successive 2n + n hybridization events between occasional unreduced egg cells from the diploids with reduced pollen from the polyploids. Once a new polyploid was established, it would reproduce clonally and very efficiently by apomixis. Occasional dihaploid formation in the apomictic tetraploids closes the cycle bringing genes from tetraploid to diploid levels. This diploid-tetraploid-dihaploid cycle strategy would allow the effective spread of the best adapted genotypes.

A second evolutionary system was suggested for species of the genus *Paspalum* (Norrmann et al. 1989, Quarin 1992). The components of this system are diploids, rare triploids and tetraploids. Diploids are sexual outbreeders, but they are able to develop occasional aposporous embryo sacs. Quarin

(1992) suggested that autotetraploids may arise in a two-step course: an occasional unreduced egg cell of a diploid plant, fertilized by a reduced sperm nucleus of a diploid forms a triploid (2n, 2x + n, x = 3x). The triploid in turn could produce a BIII offspring in combination with pollen of the surrounding diploids (2n, 3x + n, x = 4x). Since the species are perennial, a rare triploid plant included within a diploid or a mixed (2x – 4x) population could have recurrent chances to give rise to new autotetraploid genotypes.

These evolutionary interploidy gene flow models assume a higher variability rate in tetraploid clonal populations sympatric to diploid sexual individuals than in allopatric ones. Results obtained in this work suggest that the coexistence of sexual diploids and apomictic tetraploids could boost the generation of variability in apomictic systems. Because the genotypes from both tetraploid populations studied (R4X and C4X) can be considered obligate apomicts (all individuals generated from open pollinations were maternal in progeny tests), a very low rate of variability due to occasional mutation or remnants of sexuality was expected. However, the population R4X showed a variability level significantly higher than population C4X, which could be explained considering the interaction with the diploids growing in proximity. The two-step model suggested by Quarin (1992) seems more likely to occur than the diploid-tetraploid-dihaploid cycle found in *Dichanthium* by De Wet (1968), since the production of new tetraploids from apomictic triploids was experimentally verified in *P. notatum* and other species of the genus (Quarin et al. 1989). The occasional occurrence of triploids in natural populations of *Paspalum notatum* (Gould 1966, Quarin et al. 1989, Tischler and Burson 1995) favours this hypothesis. In fact, triploid individuals were isolated near El Sauce town, 400 km south from the Riachuelo area (Quarin et al. 1989). An exhaustive screening and chromosome counting survey should be performed in the future within the Riachuelo area to detect the presence of rare triploids. On the other hand, dihaploidization events have never been observed in *Paspalum*, and all attempts to obtain experimental dihaploids repeatedly failed in our laboratory (unpublished data).

The higher variability in the R4X population in respect to the C4X group suggest that some variability might be pumped up from diploids growing close to R4X. However, if there was a periodic generation of novel tetraploid clones from the interaction between R4X individuals and R2X diploids one would expect the R4X individuals to be interspersed through the R2X individuals. The tree shows just the opposite, being all the tetraploid individuals monophyletic with the exception of one (R4X30), that presented a unique genetic constitution. This observation may indicate that triploidization events could be only occasional. Almost all tetraploids studied could have been originated from a single triploid that suffered several 2n + n hybridization episodes. Certainly, triploids occur very infrequently in natural populations (Gould 1966, Quarin et al. 1989, Tischler and Burson 1995). The fact that the plant R4X30 was the only tetraploid phenotypically classified as a diploid (it was collected within a slope where diploids and tetraploids coexists, see Materials and Methods) might indicate that it could have been originated through a more recent and independent tetraploidization incident. On the other hand, tetraploids growing in Chaco (C4X) appear to be more related to the other tetraploids than to diploids. It is important to note that the Chaco population grows alongside a road and could have migrated there from the Riachuelo area through the frequent transport of cattle. However it is evident that once installed in Chaco the genetic structure of the population remained relatively uniform probably due to its geographical isolation and its clonal mode of reproduction. Further studies including more populations along with phylogenetic markers that allowed the population history as well as gene flow to be assessed would be a helpful avenue to pursue in order to evaluate the validity of the models supported by our observations in other related systems.

Species reproducing within agamic systems appear to have developed a very efficient cohesive evolutionary unit that balance adaptability and productivity by forcing genes to move across different ploidy levels. In this context apomixis does not completely prevent variation but multiplies very effectively certain products, providing the organisms with the possibility of a fine equilibrium between constancy and change. New polyploid clones may be produced continuously, not only because the character is seldom completely obligate but also because gene flow can occur between diploids and polyploids. Natural selection will act on the polyploids and superior clones will survive. These better adapted clones will conserve through apomictic behaviour avoiding segregational load, and they will continue to exist as long as they can meet the challenge from the sexuals and other apomictic clones. Eventually they can spread and invade other niches.

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