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Localization of the 5S and 45S rDNA Sites and cpDNA Sequence Analysis in Species of the Quadrifaria Group of *Paspalum* (Poaceae, Paniceae)

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• *Background and Aims* The Quadrifaria group of *Paspalum* (Poaceae, Paniceae) comprises species native to the subtropical and temperate regions of South America. The purpose of this research was to characterize the I genomes in five species of this group and to establish phylogenetic relationships among them.
• *Methods* Prometaphase chromatin condensation patterns, the physical location of 5S and 45S rDNA sites by fluorescence *in situ* hybridization (FISH), and sequences of five chloroplast non-coding regions were analysed.
• *Key Results* The condensation patterns observed were highly conserved among diploid and tetraploid accessions studied and not influenced by the dyes used or by the FISH procedure, allowing the identification of almost all the chromosome pairs that carried the rDNA signals. The FISH analysis of 5S rDNA sites showed the same localization and a correspondence between the number of sites and ploidy level. In contrast, the distribution of 45S rDNA sites was variable. Two general patterns were observed with respect to the location of the 45S rDNA. The species and cytotypes *Paspalum haumanii* 2*x*, *P. intermedium* 2*x*, *P. quadrifarium* 4*x* and *P. exaltatum* 4*x* showed proximal sites on chromosome 8 and two to four distal sites in other chromosomes, while *P. quarinii* 4*x* and *P. quadrifarium* 2*x* showed only distal sites located on a variable number of small chromosomes and on the long arm of chromosome 1. The single most-parsimonious tree found from the phylogenetic analysis showed the Quadrifaria species partitioned in two clades, one of them includes *P. haumanii* 2*x* and *P. intermedium* 2*x* together with *P. quadrifarium* 4*x* and *P. exaltatum* 4*x*, while the other contains *P. quadrifarium* 2*x* and *P. quarinii* 4*x*.
• *Conclusions* The subdivision found with FISH is consistent with the clades recovered with cpDNA data and both analyses suggest that the Quadrifaria group, as presently defined, is not monophyletic and its species belong in at least two clades.

Key words: Grasses, *Paspalum*, Quadrifaria group, cpDNA sequence analysis, molecular evolution, double-target FISH, rRNA genes, karyotype, prometaphase chromatin patterns.

INTRODUCTION

The genus *Paspalum* (Poaceae, Paniceae) includes between 350 and 400 species most of them native to tropical and subtropical regions of the Americas (Chase, 1929; Clayton and Reinvoize, 1986). Several of the species are of economic importance for forage, turf and ornamental purposes (Burson and Bennett, 1971). Ploidy levels in the genus range from diploid to octoploid (De Moraes *et al*., 1974) and practically all *Paspalum* species have a basic chromosome number of $x = 10$ (Burton, 1940).

Based on morphological characteristics of reproductive and vegetative organs Chase (1929) subdivided the genus into informal groups. The Quadrifaria group of *Paspalum* was first described by Barreto (1954, 1966), and comprises at least ten species which were originally included in the Virgata group of Chase (1929). They are all native to the subtropical and temperate regions of South America (Barreto, 1966), and sexual diploids and apomictic polyploids are known for almost all of them (Norrmann *et al*., 1989).

Meiotic chromosome pairing in interspecific hybrids of diploid *P. quadrifarium* Lam., *P. haumanii* Parodi, *P. intermedium* Munro, *P. quarinii* Morrone & Zuloaga and *P. densum* Poir. showed that they all contained a particular form of the I genome (Quarín and Norrmann, 1990; Caponio and Quarín, 1993). This genome has been described in other groups such as Virgata and Dilatata in which several allotetraploid species have been assigned the genomic formula IIJJ (Burson, 1983). In contrast to the I genome, the only proposed source of the J genome is *P. juergensii* (Burson, 1983). Since the II genomic formula has been assigned to all the diploid Quadrifaria cytotypes analysed to date (Quarín and Norrmann, 1990; Caponio and Quarín, 1993), a species from this group may be the source of the I genome for the Dilatata and Virgata polyploids. To advance in the study of the affinities among the polyploid groups and the diploid I genome donors, the different I genomes present in the Quadrifaria species must be characterized further.

From the cytogenetic point of view, chromosome pairing is not likely to provide further insight into the phylogenetic relationships among diploid and polyploid species with

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TABLE 1. *Paspalum species and cytotypes analysed, with respective chromosome number, collection number, provenance, and number and position of 5S and 45S rDNA sites*

Species	2*n*	Collection no.	Provenance	No. and position of 5S rDNA sites*	No. and position of 45S rDNA sites*
P. intermedium	20	V11802	18 km south from Dourados, MGS, Brazil	2 (1p)	4: 2 (5t) + 2 (8p)
P. haumanii	20	Q3860	Paso Lucero, Corrientes, Argentina	2 (1p)	4: 2 (5t) + 2 (8p)
P. quadrifarium	20	NA2623	R5 km 491, Rivera, Uruguay	2 (1p)	5: 2 (5t) + 1 (1t) + 1 (7t) + 1 (10t)
P. quadrifarium	40	NA7664	Cañada Ibañez, Cerro Largo, Uruguay	4 (1p)	7: 4 (5t) + 2 (8p) + 1 (t)
*P. quarinii***	40	BRA-020923	São Miguel das Missões, RGS, Brazil	4 (1p)	7: 2 (both ends of one chromosome 1) + 2 (5t) + 3 (t)
P. exaltatum	40	BRA-022101	Caseiros, RGS, Brazil	4 (1p)	6: 4 (8p) + 2 (t)

* The position of the site was given by the chromosome order on a monoploid complement (1–10) followed by its location as proximal (p) or terminal (t).
** = *P. brunneum* auct. non Mez (Morrone and Zuloaga, 2000).

I genome since some degree of multivalent formation in these polyploids is to be expected. On the other hand, the mitotic chromosomes in *Paspalum* are small and morphologically similar, making their karyotypic differentiation very difficult. An alternative has been the use of the prometaphase stage in which uneven condensation of the chromatin has allowed chromosome identification and karyotyping in several plant genera (Fukui and Iijima, 1991; Nakamura *et al*., 2001). Distinctive patterns of chromatin condensation in prometaphase were also clearly observed in *Paspalum* chromosome squashes allowing the construction of karyotypes in a triploid and a tetraploid cytotype of *P. quadrifarium* and a segmental allopolyploid origin was suggested for both (Speranza *et al*., 2003). Further refining of the cytogenetic approach can be achieved by analysing other chromosome markers.

Fluorescence *in situ* hybridization (FISH) of repetitive DNA sequences has been used as a tool for karyotype and genome analyses of a large number of plant species. The repetitive and tandemly organized ribosomal rDNA genes, 5S rDNA and 45S rDNA, are localized at one or more sites per chromosome set, and their characteristic positions provide useful markers for chromosome and genome identification. They have been used in several grasses such as *Triticum* and *Hordeum* (Jiang and Gill, 1994), *Oryza* (Shishido *et al*., 2000), *Sorghum* (Sang and Liang, 2000), *Thinopyrum* (Brasileiro-Vidal *et al*., 2003), *Festuca* (Harper *et al*., 2004), providing useful information about evolutionary and phylogenetic relationships between species.

A molecular phylogeny is not available for *Paspalum* yet, and it is likely that some of the morphological groups recognized to date, would need to be at best re-evaluated when sequence information becomes available. Because of the prevalence of polyploidy and hybridization in the genus, the use of nuclear sequences does not seem appropriate as a first approach given the technical difficulties implied in isolating them and the reticulate evolutionary pattern of the group (Sang, 2002). Several universal primers are available from the literature for highly variable, non-coding chloroplast regions (Demesure *et al*., 1995; Soltis and Soltis, 1998). These regions can provide a useful phylogenetic signal to analyse the relationships among the diploid species that have the different variants of the I genome.

In the present study, prometaphase chromatin condensation patterns, the physical location of 5S and 45S rDNA sites by double-target FISH, and sequences of five chloroplast non-coding regions were used to characterize the I genomes of some diploid and polyploid cytotypes of five species of the Quadrifaria group of *Paspalum* and to establish phylogenetic relationships among them. An attempt was also made to localize the heterochromatin blocks using the base specific fluorochromes chromomycin A_3 (CMA) and 4′,6-diamidino-2-phenylindole (DAPI), which bind preferentially to GC- or AT-rich chromosome regions, respectively (reviewed by Guerra, 2000).

MATERIALS AND METHODS

Plant material

Six accessions belonging to five species of the Quadrifaria group of *Paspalum* were used for cytogenetic analysis: *P. quadrifarium* Lam., *P. haumanii* Parodi, *P. intermedium* Munro, *P. quarinii* Morrone & Zuloaga and *P. exaltatum* K. Presl. Plants were obtained from collections growing at the Facultad de Agronomía, Montevideo, Uruguay, or at Embrapa-Cenargen, Brasília, Brazil (Table 1). The other known source of I genomes, *Paspalum rufum* Nees (Virgata group), and *Axonopus rosengurttii* (MCM 05, Montevideo, Uruguay) were included as outgroups for the cpDNA phylogenetic analysis.

Pretreatment and fixation

Selected root tips were obtained from plants growing in pots and pretreated with 2 mM 8-hydroxyquinoline for 2 h at room temperature and 2 h at 4 °C. They were fixed in Carnoy 3 : 1 (ethanol : acetic acid) solution for at least 24 h at room temperature and then stored at −20 °C until used.

Chromosome preparation for karyogram analysis

The protocol used for chromosome preparations for karyotype analysis followed that of Speranza *et al*. (2003). Fixed root tips were rinsed in buffer (40 mM citric acid, 60 mM sodium citrate) for 15 min and digested for 4 h at 37 °C with a combination of 3 % (w/v) cellulase (Calbiochem, San Diego, CA, USA), 1 % (w/v), cellulase Onozuka RS (Yakult Pharmaceutical, Japan) and 4 % (v/v) pectinase (Sigma, St Louis, MO, USA). Root tips were squashed in 45 % acetic acid and coverslips were removed by freezing in liquid nitrogen. The slides were washed in

70 % acetic acid at 40 °C for 5–10 min to remove the remaining cytoplasm, stained with 2 % lacto-propionic (1 : 1) orcein (Sigma), and sealed. The construction of the karyograms followed Speranza *et al*. (2003).

Cells were photographed on an Olympus New Vanox Microscope and highly amplified conventional prints were digitized and treated with Corel Draw version 11 for processing. Idiogram construction was based on the analysis of at least five well-spread prometaphases. Measurements were made on the public domain software Image J 1.28u (http://rsb.info.nih.gov/ij/Java1.3.1_03).

Chromosome preparation for fluorochrome banding and FISH

For these procedures, chromosome preparations were performed according to Cornélio *et al*. (2003). Fixed root tips were washed in distilled water for 15 min and digested in 2 % cellulase Onozuka R-10 (Serva) and 20 % pectinase (Sigma) for 4 h at 37 °C. Root tips were squashed in 45 % acetic acid and coverslips were removed by freezing in liquid nitrogen and then slides were air-dried. The best slides were selected after a brief stain with DAPI/ glycerol (v/v), destained in Carnoy 3 : 1 for 30 min and dehydrated in 100 % ethanol for at least 2 h. Slides were stored at −20 °C until used.

For CMA/DAPI staining the slides were aged for 3 d, stained with 0·5 mg ml^{-1} chromomycin A_3 (CMA; Sigma) for 1 h and 2 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for 30 min and mounted in 1 : 1 (v/v) McIlvaine's pH 7 buffer/glycerol. Images were acquired using a Cohu-CCD video camera attached to a DMLB Leica epifluorescence microscope, and combined using the Leica Qfish software. Final processing was made using Corel Draw version 11. After image acquisition, the coverslips were removed, the slides were destained in ethanol–acetic acid (3 : 1) for 30 min, dehidratated in 70 % ethanol overnight, air dried and stored at −20 °C until used for FISH.

DNA probes and labelling

Two DNA probes, R2 and D2, were used in the FISH experiments. Probe R2 is a 6·5-kb fragment containing an 18S–5·8S–25S rDNA repeat unit (including internal transcribed spacers ITS1 and ITS2 and a short 5′ fragment of the intergenic region from *Arabidopsis thaliana* (Wanzenböck *et al*., 1997). Probe D2, is a 500-bp fragment of the 5S rDNA gene repeated unit from *Lotus japonicus* (Pedrosa *et al*., 2002). Both probes were labelled by nick translation (Gibco) with digoxigenin-11-dUTP (Roche, Molecular Biochemicals, Sussex, UK) and biotin-11-dUTP (Sigma), respectively.

Double-target FISH

The *in situ* hybridization technique followed Heslop-Harrison *et al*. (1991) with the modifications introduced by Pedrosa *et al*. (2001). Hybridization mixture consisted of 60 % (v/v) formamide, 5 % (p/v) dextran sulfate, 2 × SSC, 0·01 % salmon sperm DNA and between 20 and 50 ng μL^{-1} of each labelled probe. The mixture was preheated at 75 °C for 10 min and kept on ice for 5 min. Then 30 μL of the mixture was added to each slide and chromosomes together with the probes were denatured on a hot plate at 80 °C for 10 min. Slides were then transferred to a humid chamber for overnight hybridization at 37 °C. Following hybridization, slides were given a stringent wash with 0·1× SSC at 42 °C for 5 min and washed in 2× SSC. Digoxigenin-labelled probe, R2, was detected with FITC (fluorescein isothiocyanate; Roche), and biotin-labelled probe, D2, was detected with TRITC (tetramethyl rhodamine isothiocyanate; Dako). Slides were counterstained and mounted with 2 μg mL^{-1} DAPI in Vectashield (Vector). The images were acquired as described before.

Sequencing

Silica-gel dried leaves of the same individuals used for cytogenetic and FISH analysis were used to extract DNA with a Sigma Genelute™ kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. Universal primers (Taberlet *et al*., 1991) were used to amplify the *trn*L(UAA) intron (primers C and D of Taberlet *et al*., 1991), the PsbA-*trn*H spacer (Sang *et al*., 1997) and the *atpB–rbcL* spacer (Hodges and Arnold, 1994). Primers for the *trn*G(UCC) intron were obtained from Shaw *et al*. (2004). The *trn*L(UAA)–*trn*F(GAA) spacer could not be amplified directly from genomic DNA in any of these *Paspalum* species using primers E and F (Taberlet *et al*., 1991); therefore, E-F fragments were obtained by nested PCR with primers E and F using 1 : 1500 to 1 : 5000 dilutions of the C-F fragments as template. All PCR amplifications were carried out in 25-μL reactions containing 0·4 units of NEB Taq polymerase (New England Biolabs, Beverly, MA, USA) 1·5 mM $MgCl_2$, 0·4 μM of each primer and 0·1 mM of each dNTP in the manufacturer's buffer. Amplification was carried out in a Biometra® T3 Thermoblock with the same programme for all fragments. The programme consisted of an initial 5 min at 95 °C, followed by one cycle of 1 min at 94 °C, 1 min at 58 °C and 2 min 30 s at 72 °C, the annealing temperature was decreased by 1 °C for six cycles and then 32 additional cycles were carried out with an annealing temperature of 52 °C followed by a final elongation step of 5 min at 72 °C. PCR products were cleaned with Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) and diluted to approx. 1 ng μL^{-1} before sequencing.

All regions were sequenced in both directions on a CEQ 8000 capillary sequencer (Beckman-Coulter, Fullerton, CA, USA) using one-quarter reaction volumes with the addition of 80 mM Tris and 2 mM $MgCl_2$ (pH 9) to complete the volume of a full reaction. The same PCR primers were used for sequencing, except for primer E because a poly-A tract located 65 bp from its end prevented further sequencing in most materials. Primer E2 (5′-AAAGGAGTGCGACGAGAAC-3′) was designed starting at position 127, just internal to the poly-A tract. This primer was designed with Primer 3 (Rozen and Skaletsky, 2000) based on the sequences obtained with primer F for this region.

The sequences were edited manually using Sequencher™ (V4·1·4, Genecodes, AnnArbor, MI, USA) and all

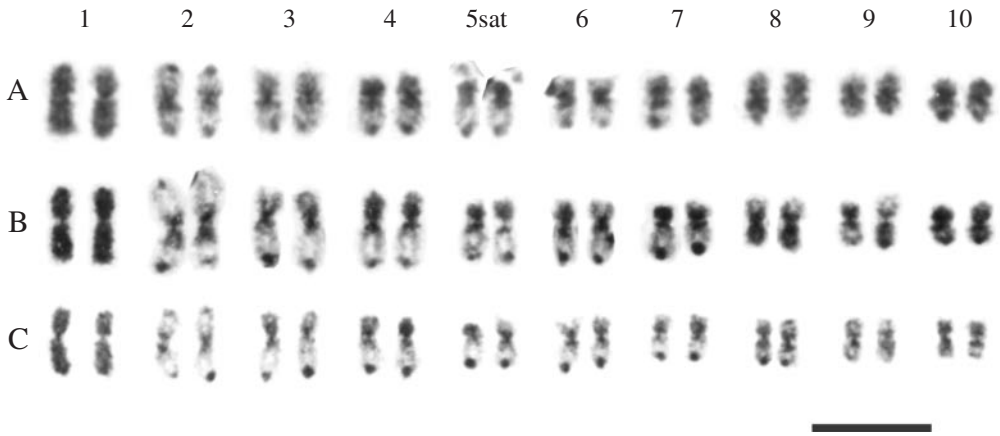

FIG. 1. Karyograms of diploid cytotypes of (A) *P. intermedium*, (B) *P. haumanii*, and (C) *P. quadrifarium* based on orcein stained prometaphase chromosomes. Scale bar = 10 μm.

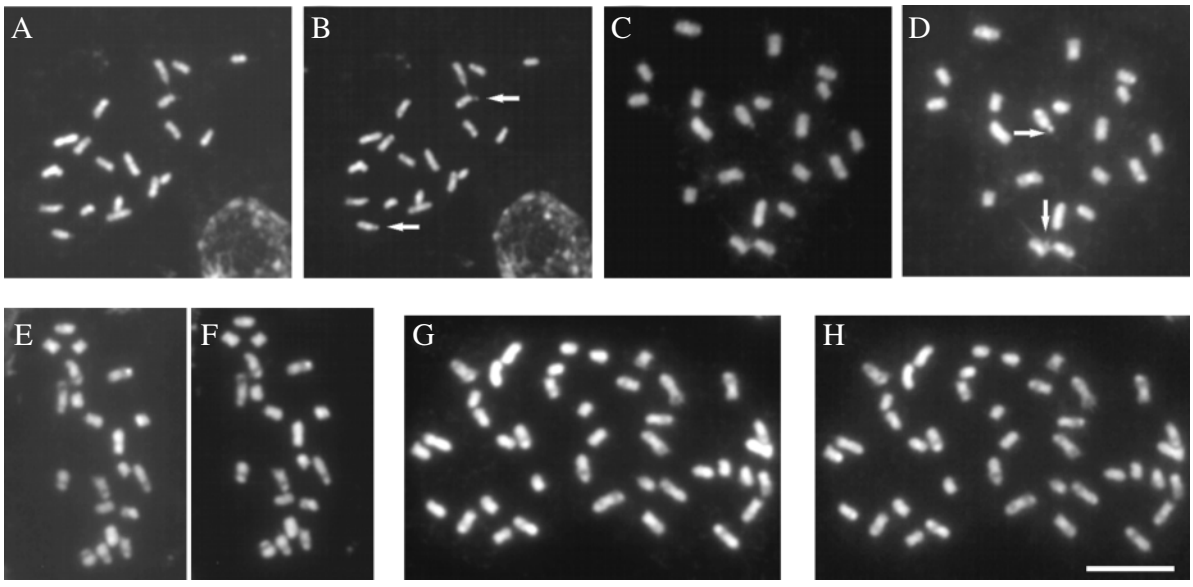

FIG. 2. Prometaphase chromosomes of *P. haumanii* 2*x*, *P. intermedium* 2*x*, *P. quadrifarium* 2*x* and *P. quadrifarium* 4*x* after DAPI (A, C, E and G) and CMA staining (B, D, F and H). Arrows point out satellites. Scale bar = 10 μm.

ambiguous end regions removed. The resulting partial sequences were prealigned with the Clustal-W (Thompson *et al*., 1994) algorithm included in BioEdit (V 5.0.6; Hall, 1997) and the resulting alignments were manually adjusted. All sequences and alignments were submitted to Genbank (Accession nos. AY941120–AY941159). Parsimony analyses for all five regions combined in a single matrix were carried out in PAUP* (V 4.0b10, Swofford) using an exhaustive search; bootstrap analysis was performed with 10 000 replicates and 100 SAR per replicate.

RESULTS

Chromatin condensation patterns and fluorochrome banding

The karyotypes of the diploid cytotypes of *Paspalum intermedium*, *P. haumanii* and *P. quadrifarium* are shown in Fig. 1. The three species presented symmetric karyotypes with metacentric and submetacentric chromosomes. The general features of prometaphase chromatin condensation patterns obtained after orcein staining were basically the same for the three diploid cytotypes and allowed the identification of homeologous chromosomes. CMA/DAPI double staining revealed the same condensation pattern observed after orcein staining (Fig. 2). CMA-stained chromosomes exhibited more contrasted bands than DAPI. Chromosome 1 presented an entirely homogenous chromatin condensation pattern in both arms. Chromosomes 2 and 8 showed condensed proximal and terminal regions. Condensation in the other chromosomes includes the proximal regions and a characteristic distal region of the long arm, while their short arms are entirely or almost entirely condensed. Only one submetacentric satellite–chromosome pair (chromosome 5) was observed, with the satellite located in the short arm (Fig. 1A; Fig. 2B and D). The satellites were not stained with DAPI (Fig. 2A and C).

In prometaphase and metaphase, satellites are frequently found separated from the chromosomes and also became lost (Fig. 1C). In the tetraploids analysed with CMA/DAPI no secondary constriction was observed (Fig. 2H). After *in situ* hybridization, the DAPI banding pattern was not only carefully preserved in all species but even more contrasted than before, and it was very useful to identify the chromosomes labelled by the probes.

Number and distribution of 5S rDNA sites

Figure 3 shows the results of the double-target *in situ* hybridization with the 5S rDNA and 45S rDNA probes to mitotic chromosomes of the Quadrifaria species and cytotypes. The number and localization of the rDNA sites are summarized in Table 1.

The three diploid species, *P. quadrifarium*, *P. haumanii* and *P. intermedium*, have one pair of 5S rDNA sites (Fig. 3A–C) and polyploid plants were found to have the expected number of these loci (Table 1). Thus tetraploid cytotypes of *P. quarinii*, *P. quadrifarium* and *P. exaltatum* had four hybridization sites (Fig. 3D–F). The signals in all species and cytotypes were detected in a proximal location on the short arm of chromosome 1, which is characterized by its homogeneous chromatin condensation pattern in prometaphase.

Number and distribution of 45S rDNA sites

The *in situ* hybridization analysis revealed four sites for the 45S rDNA in the species *P. haumanii* and *P. intermedium*: two strong large-sized signals were localized at the end of the short arms of SAT chromosome 5 and a minor pair of sites was visible at the condensed proximal region of chromosome 8 (Figs 3A and C and 4B and C). For the diploid cytotype of *P. quadrifarium*, a different organization was observed. Five 45S rDNA sites were detected and all of them were located on the terminal end of the short arms of the chromosomes. Strong signals were localized at the end of the short arm of both homologous SAT chromosomes 5, and three minor sites on the short arms of only one member of chromosome pairs 1, 7 and 10. The signals in the homologous chromosome of these pairs were not detected (Figs 3B and 4A).

In the tetraploid cytotype of *P. quarinii*, a total of seven 45S rDNA sites were observed (Fig. 3D). Two of these were located at both terminal regions of only one member of chromosome pair 1. It is interesting to note that these two sites were unequal in size; the site at the end of the long arm was larger than the one found at the shorter one. Two other sites were observed at the terminal regions of two chromosomes that resemble chromosome 5 in diploid species. The other three sites were distributed at terminal regions of three small chromosomes.

The tetraploid individual of *P. exaltatum* had six 45S rDNA sites (Fig. 3F): four major sites were located in the condensed proximal regions of chromosomes that present a chromatin pattern similar to chromosome pair 8 of the diploid species, while the other two minor signals were located at the terminal region of two small chromosomes. No signal was detected at the chromosome corresponding to the number 5 of diploid species.

For the tetraploid cytotype of *P. quadrifarium* seven signals were observed and its hybridization pattern did not represent the exact doubling of the diploid cytotype of *P. quadrifarium* (Fig. 3E). Four 45S rDNA sites were located on the distal region of the short arms of two chromosome pairs corresponding to chromosome 5 of diploids, a minor site was observed on the terminal region of a small chromosome, and two signals were detected in the proximal region of chromosome 8.

cpDNA sequences

Separate phylogenetic analyses were conducted for each sequenced region and no conflicts were found for supported branches. Each gene supported a subset of the nodes; however, the *trn*L–*trn*F spacer sequence matrix alone produced almost all the relationships found for the combined data set, except with bootstrap supports ranging from 60 to 94 %. All the data were combined in a single matrix for further analysis. The resulting combined matrix consisted of a total length of 2367 bp positions, 74 of which were variable and 23 parsimony informative. A single most-parsimonious tree of length 104 (CI = 0·9327, RC = 0·7772) was found for this set of sequences (Fig. 5). Bootstrap values for all nodes ranged between 77 and 100 % with one short branch showing 59 %. The tree shows the Quadrifaria species analysed here partitioned in two clades, one of them includes *P. haumanii* and *P. intermedium* together with the tetraploid cytotypes of *P. quadrifarium* and *P. exaltatum*, while the other contains the diploid *P. quadrifarium* and the tetraploid *P. quarinii*.

DISCUSSION

Karyograms based on the differential prometaphase chromatin condensation pattern

The prometaphase chromosome condensation patterns observed here allowed the identification of most homologous chromosomes and the karyotype construction of the diploid cytotypes of *Paspalum haumanii*, *P. intermedium* and *P. quadrifarium*. This banding pattern was also highly conserved in the tetraploid accessions investigated here and in the polyploid cytotypes of *P. quadrifarium* reported by Speranza *et al*. (2003). These patterns were not influenced by the dyes used (CMA, DAPI or orcein) or by the FISH procedure, therefore the bands are not necessarily related to heterochromatin or to GC- and AT-rich regions. They most probably represent the longitudinal variation in the chromosome condensation at the prophase and prometaphase (for a discussion of heterochromatin and condensation patterns, see Guerra, 1988). Eventually, this uneven chromatin condensation allowed the identification of almost all the chromosome pairs that carried the rDNA signals in the species analysed here.

FISH pattern of the 5S rDNA sites

The number and location of the 5S rDNA sequence appeared to be conserved among the Quadrifaria species

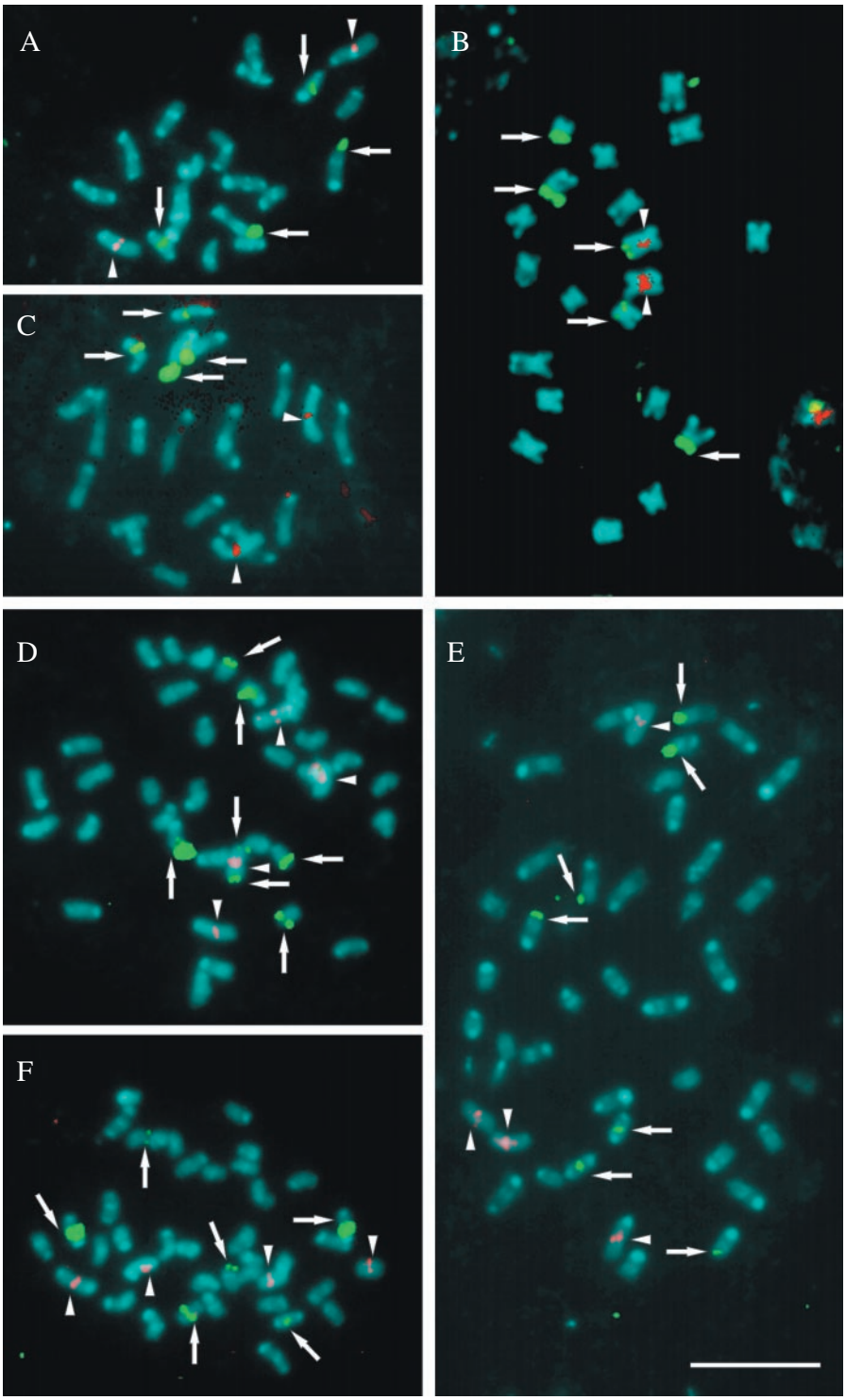

FIG. 3. Fluorescence *in situ* hybridization with 45S (green) and 5S (red) rDNA probes, in prometaphase chromosomes of (A) *P. haumanii* 2*x*, (B) *P. quadrifarium*, (C) *P. intermedium* 2*x*, (D) *P. quarinii* 4*x*, (E) *P. quadrifarium* 4*x*, and (F) *P. exaltatum* 4*x*. Arrowheads indicate 5S rDNA sites while arrows indicate 45S rDNA sites. Note, in B and D, 45S and 5S rDNA sites located on chromosome 1. Chromosomes were counterstained with DAPI (blue). Scale bar = 10 μm.

and cytotypes. As seen for other plant genera (D'Hont *et al*., 1998; Adams *et al*., 2000; de Melo and Guerra, 2003), a correspondence between the number of 5S rDNA loci and ploidy level was found for the tetraploid specimens of *P. quadrifarium, P. quarinii* and *P. exaltatum*, which all exhibit the four expected signals. Such a conservation of the 5S rDNA loci is not a common feature in grasses, where variability in number, location or both has been reported

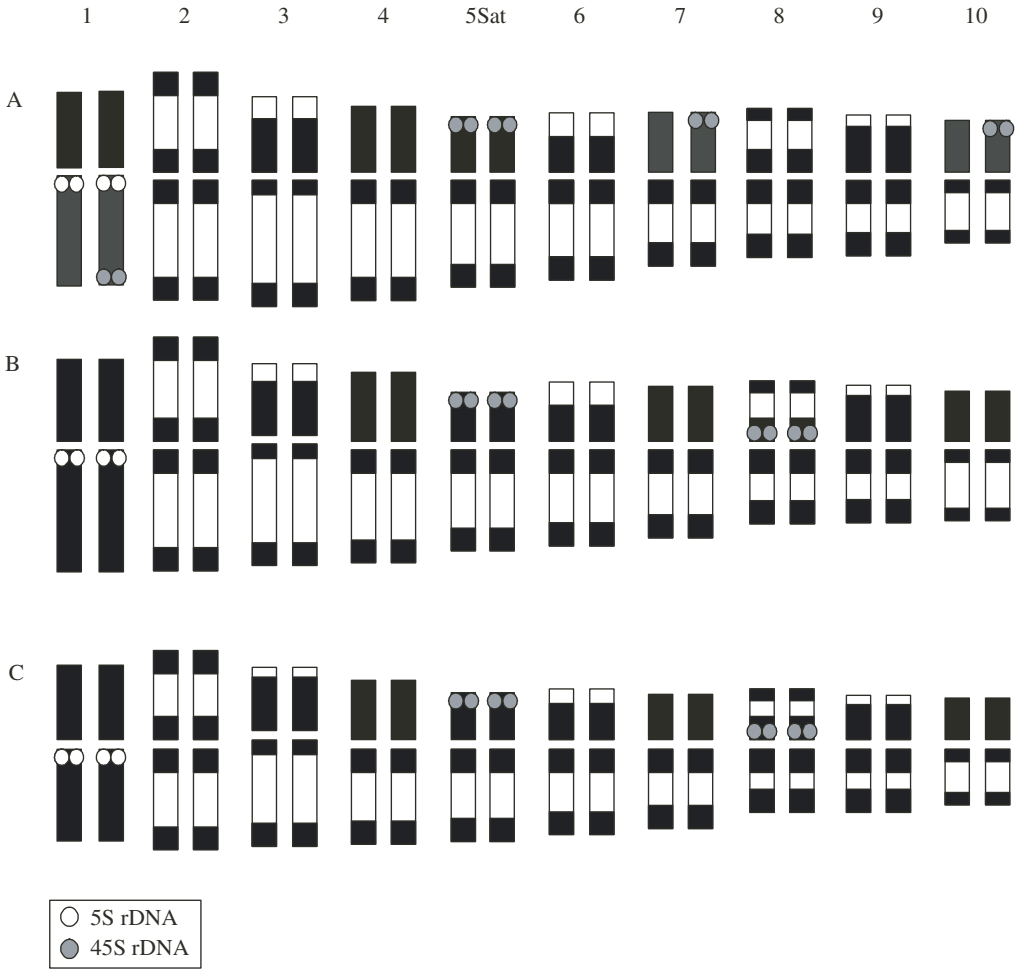

FIG. 4. Idiograms of (A) *Paspalum quadrifarium* 2*x*, (B) *P. haumanii* 2*x*, and (C) *P. intermedium* 2*x*. Distribution of 5S rDNA sites (white dots) and 45S rDNA sites (grey dots) is indicated.

(Thomas *et al*., 1996; Shishido *et al*., 2000; Taketa *et al*., 2001; Harper *et al*., 2004).

Variability in the number and location of 45S rDNA sites among species

In contrast to the 5S rDNA, several differences in the location of 45S rDNA loci were detected. The large number of heterozygous pairs found in the diploid individual of *P. quadrifarium* is particularly remarkable. This unexpected variation may be attributed to the fact that diploid cytotypes of *P. quadrifarium* are sexual and self-incompatible (Norrmann *et al*., 1989). The population from which this individual was collected most probably is polymorphic for the presence of 45S rDNA sites on chromosomes 1, 7 and 10, and provides evidence of the high mobility of the 45S rDNA within a population. Polymorphisms for 45S rDNA sites have been also reported in other grasses such as *Lolium* (Thomas *et al*., 1996) and *Hordeum* (Taketa *et al*., 1999, 2001). In the Quadrifaria case, the general structural similarity among the karyotypes of all the species studied suggested that major chromosome rearrangements are not frequent in this group. Therefore, mechanisms such as transposon mobility and amplification of cryptic minor rDNA sites by unequal crossing-over rather than chromosomal rearrangements may be involved in the origin of the variation of the 45S rDNA. The possibility that nucleolar organizer regions (NORs) in *Paspalum* may have moved via magnification of minor loci consisting of few repeats and deletion of major inactive sites, as proposed by Dvořák (1989) and Dubcovsky and Dvořák (1995) for the Triticeae, may be a serious concern regarding the use of ITS sequences for phylogeny reconstruction (Dubcovsky and Dvořák, 1995; Adams *et al*., 2000).

Two general patterns were observed in the Quadrifaria accessions with respect to the location of the 45S rDNA. The species and cytotypes *P. haumanii* 2*x*, *P. intermedium* 2*x*, *P. quadrifarium* 4*x* and *P. exaltatum* 4*x* showed proximal sites on chromosome 8 and a variable number of distal sites, while *P. quarinii* 4*x* and *P. quadrifarium* 2*x* showed only distal sites located on a variable number of small chromosomes and on the long arm of chromosome 1.

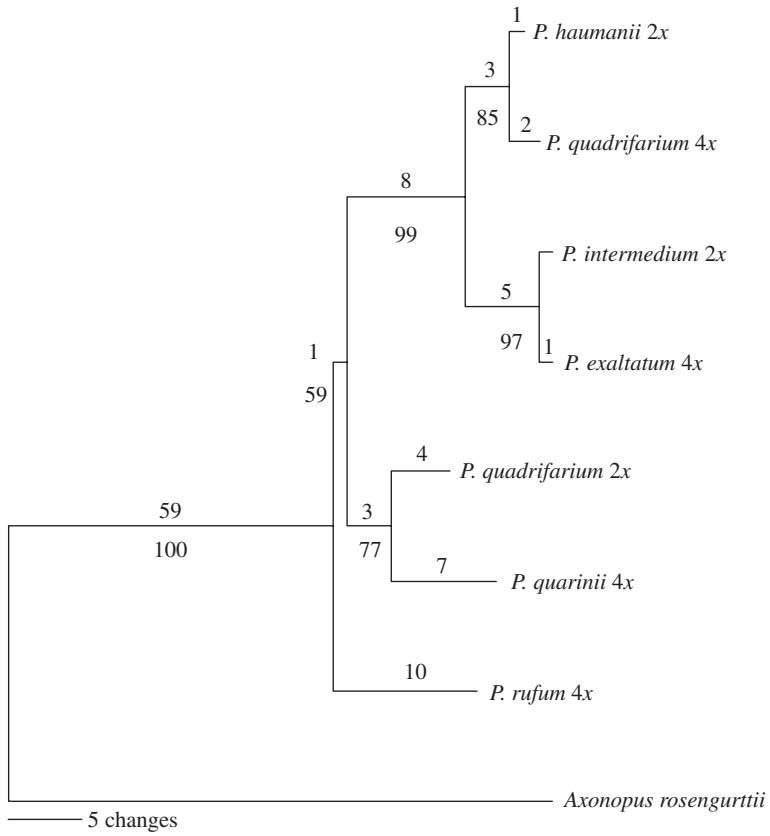

FIG. 5. The single most-parsimonious tree found using an exhaustive search from the phylogenetic analysis of five chloroplast non-coding regions (see text). Numbers above the branches indicate the number of base substitutions and numbers below the branches are bootstrap support percentages out of 10 000 replicates.

cpDNA sequence analysis

The subdivision between the Quadrifaria species found with the FISH analysis is consistent with the two clades recovered with cpDNA data. These clades, in turn, are congruent with those found in a preliminary cpDNA sequence analysis of about 70 *Paspalum* species which includes the ones studied in this paper (P. Speranza and G. Rua, unpubl. res.). This preliminary analysis also shows that diploid *P. quadrifarium* is sister to a clade containing *P. quarinii* and a wide array of species including the Paniculata group, a proposed source of the J genomes to the Dilatata group (Burson, 1978). From the morphological point of view, the Quadrifaria group includes species with very similar characteristics. Differences among *P. quadrifarium*, *P. exaltatum* and *P. haumanii* for example, are mostly quantitative (Barreto, 1966); however, *P. haumanii* and *P. intermedium* are extremely large bunchgrasses often reaching heights of 2–3 m, while the species included in the other clade never reach such size (Barreto, 1966). The information presented here suggests that the Quadrifaria group, as presently defined, is not monophyletic and its species belong in at least two clades which may in turn be paraphyletic to other morphological groups. More species need to be analysed in order to identify stable synapomorphies for the clades that make up the Quadrifaria group.

Origin of the polyploid cytotypes

The tetraploid plant of *P. quadrifarium* used here is likely to be a hybrid between species belonging to the two main clades into which the Quadrifaria group appears to be divided. The presence of two distinct genomes reported by Speranza *et al*. (2003) in the same individual, suggested a segmental allopolyploid origin. That conclusion, based on the lack of multivalent formation during meiosis and prometaphase chromosome analysis, is further supported here by the 45S rDNA hybridization pattern of this plant – only two signals in the proximal region of chromosome 8. Moreover, this plant morphologically resembles diploid *P. quadrifarium* but its chloroplast genome is more similar to that of *P. haumanii*. This provides additional support for an allopolyploid origin for this cytotype.

The chloroplast haplotype of the tetraploid individual of *P. exaltatum* used here, was closely related to that of *P. intermedium*; however, *P. exaltatum* is morphologically very similar to *P. haumanii*, which also suggests a hybrid origin for this plant. The presence of four 45S rDNA proximal signals on chromosome 8 in this tetraploid *P. exaltatum* indicates that it most probably originated by hybridization between two species with a karyotype similar to those of *P. intermedium* and *P. haumanii*.

Differences between the expected and observed number of 45S rDNA loci was also detected in the polyploid

cytotypes of *P. quadrifarium* and *P. quarinii*. There is significant evidence for genetic alteration of the genome following polyploidization (Wendel, 2000; Levy and Feldman, 2004). The loss of ribosomal genes after allopolyploidization has been seen in many plant genera (Vaughan *et al*., 1993; Thomas *et al*., 1997; Mishima *et al*., 2002). Thus, unlike the 5S rDNA sites, some 45S rDNA loci of the *Paspalum* accessions investigated here appear to have been eliminated after polyploidization.

Cytogenetic differentiation

All the diploid species analysed here have been assigned the II genome formula. Quarín and Norrmann (1990) found that for crosses between pairs of diploid cytotypes belonging to the same cpDNA clade (*P. intermedium* × *P. haumanii* and *P. quadrifarium* × *P. quarinii*), the mean number of bivalents of each hybrid was nearly 10. In the same paper it is reported that for inter-clade crosses that number was close to 8·5 and for hybrids of all four species to *P. rufum* (Virgata group), the only diploid source of I genome outside the Quadrifaria group, it was approx. 7. The mean number of bivalents in the hybrids reported by Quarín and Norrmann (1990) is then roughly proportional to the phylogenetic distances estimated in this paper for their parents based on cpDNA. However, for Seberg and Petersen (1998) chromosome pairing between two species may be a poor indicator of phylogenetic relatedness, since it has been shown to be under genetic control, and even influenced by environmental conditions (Jahuar and Joppa, 1996; Seberg and Petersen, 1998). Thus, more phylogenetic and cytogenetic data are required to assess whether chromosome pairing reflects phylogenetic distances in the genus *Paspalum* both among and within its major clades.

The evidence presented here sharply contrasts with the implicit interpretation of meiotic studies that autopolyploidy had played a major role in the evolution of the polyploid cytotypes of the Quadrifaria group (Quarín and Lombardo, 1986; Quarín and Norrmann, 1987). Instead, it appears that at least some of the polyploid apomictic cytotypes of the Quadrifaria group are part of a vast hybrid complex.

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