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Chloroplast genome differences between *Paspalum dilatatum* Poir and the related species *P. notatum* Flugge

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Abstract Chloroplast DNA (cpDNA) of *Paspalum dilatatum* and *P. notatum* was digested singly or in combination with the restriction endonucleases *Pst*I, *Pvu*II, *Sal*I, *Kpn*I and *Xho*I. Data obtained from filter hybridization experiments with barley and wheat cpDNA probes were used to construct restriction site maps of the chloroplast genomes of the *Paspalum* species. The cpDNA fragments were ordered into a circular configuration of approximately 139.3 kbp that contained two inverted repeat regions of approximately 23 kbp and a small and large single-copy region of approximately 11 kbp and 83 kbp, respectively. The cpDNA maps showed that *P. dilatatum* and *P. notatum* shared a large number of restriction sites with the proportion of shared restriction sites S = 0.90. No restriction site differences were detected in the *Kpn*I maps. Eight species-specific restriction site differences that could be used to identify the cytoplasm of each *Paspalum* species were identified in the *Pst*I, *Pvu*II, *Sal*I, and *Xho*I cleavage maps. The overall structural organization of the *Paspalum* cpDNAs is rather similar to those of most cpDNAs from other plants. The results presented in this study will be of value for exploring further phylogenetic relationships within the genus *Paspalum*.

Key words *Paspalum* · Chloroplast DNA · Restriction site maps

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Introduction

Paspalum is a large, diverse genus of more than 400 species that grow in warm, tropical and subtropical regions (Chase 1929). The genus comprises many economically important forage, turf and ornamental species. Two of the major forage species are *P. dilatatum* (dallisgrass) and *P. notatum* (bahiagrass) that are native to South America and have become important forage grasses throughout much of the warmer regions of the world including the southern United States (Burson 1991). Various morphological and cytological forms have been described for these species (Moraes-Fernandes et al. 1968; 1973). There are at least six biotypes of *P. dilatatum*, with chromosomes numbers ranging from 40 to 60, and more than a dozen forms of *P. notatum* that occur in diploid, triploid and tetraploid forms (Moraes-Fermandes et al. 1973; Hickenbick and Flores 1992). The most common form of dallisgrass, *P. dilatatum* Poir, is a pentaploid (2n = 5x = 50) in which the 50 chromosomes pair as 20 bivalents and 10 univalents (Bashaw and Forbes 1958). Common dallisgrass is apomictic, making attempts to improve it through conventional breeding difficult since it cannot be used as a female parent in interspecific crosses (Burson et al. 1973). *Paspalum dilatatum* is considered to be a natural hybrid, and synthesis of a sexual fertile type could be possible if its progenitors were known (Burson et al. 1973). *Paspalum notatum* is a perennial, rhizomatous species. The diploid cytotype, classified as *P. notatum* var '*saurae*' Parodi, is considered to have entered the United States accidently (Burton 1967). It has been assigned the genome formula NN (Burson 1981). Its chromosomes are completely homologous with the tetraploid cytotypes of *P. notatum* Flugge (Forbes and Burton 1961). Naturally occurring tetraploid forms of *P. notatum* are known to be obligate apomicts (Bashaw et al. 1970).

The phylogenetic relationships of *P. dilatatum* and *P. notatum* within the genus *Paspalum* need to be

determined in order to obtain the genetic and breeding information necessary for the improvement of these forage species. Genetic relationships between *P. dilatatum* and *P. notatum* and their individual affinities to other congeneric species have been investigated, primarily through cytogenetic studies (Bennett and Bashaw 1960; Burson et al. 1973; Quarin and Burson 1983; Burson 1985; Caponio and Quarin 1990). In this approach, cross compatibilities and genome homologies between artifically produced hybrids are assessed. While such comparisons have provided some indirect information on species relationships, the true relationships among the species have not been determined (Burson 1981). Knowledge of the genetic affinity between related species is useful for introgressing agronomic characteristics from one species to another. The chloroplast DNA (cpDNA) of most land plants has been shown to be a highly conserved molecule in terms of size, structure, gene arrangement and primary sequence and is considered ideal material for determining genetic affinities (Palmer 1987). Chloroplast DNA restriction enzyme analysis has provided information for phylogenetic studies at all levels of taxonomic hierarchies (Downie and Palmer 1992).

The objectives of this study were to (1) construct restriction site maps of the chloroplast genomes of *P. dilatatum* and *P. notatum*, (2) describe the basic differences/similarities between their chloroplast genomes and (3) compare the chloroplast genomes of *P. dilatatum* and *P. notatum* with those of closely related grasses. The results of this study will be used to investigate further phylogenetic relationships within the genus *Paspalum*.

Materials and methods

Plant material

Seed material of *P. dilatatum* (P1202388) and *P. notatum* (PI337574) were provided by the Southern Regional Plant Introduction Station, Griffin, Ga. The seeds were germinated in plastic pots under greenhouse conditions. After 3–4 weeks of growth, individual seedlings were transplanted to larger pots and grown for another 3–4 weeks to obtain sufficient leaf material for DNA extraction. Some of these plants were grown to maturity for seed increase and taxonomic identification. Voucher specimen are in the senior author's personal collection.

DNA isolation, restriction enzyme digestion and gel electrophoresis

Total DNA was isolated from either fresh or frozen leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) procedure as described previously (Pillay 1995). The DNAs of two *Paspalum* species were digested with the restriction endonuleases *Pst*I, *Pvu*II, *Sal*I, *Kpn*I and *Xho*I solely or in combination with *Pst*I and each of the other enzymes following conditions recommended by the supplier (New England Biolabs, Beverly, Mass.). These six-base recognition site enzymes have been found to cleave plastid DNA into relatively few fragments and are useful for constructing restriction site maps.

Southern transfer and DNA hybridization

Southern transfer of DNA fragments, radio-isotope labelling of cloned cpDNA probes and molecular hybridization to 13 unique probes were carried out as described in Pillay (1993). The heterologous probes used in this study included 10 *Pst*I clones of the barley chloroplast genome provided by Dr. A. Day (University of Geneva, Switzerland) and Dr. T. H. N. Ellis (John Innes Institute) and selected clones of wheat cpDNA, including B(10-18), B(2-15), B(21-8), obtained from Dr. C. Bowman (Plant Breeding Institute, Cambridge). A map illustrating the size and approximate location of these probes has been presented in a previous publication (Pillay 1993). The wheat clones were useful in determining the position of overlapping fragments especially at the junction of the large single-copy and inverted repeat regions.

Analysis of data and physical mapping

The autoradiograms were used to measure the migration distance of each hybridization band from the origin. The molecular size of the fragments was estimated by using known standards that included lambda DNA digested with *Hin*dIII and the one-kilobase ladder marker (Life Sciences, Gaithersberg, Md. USA). The *Paspalum* cpDNA fragments that hybridized to each of the clones were recorded and used to construct the physical maps by the overlap hybridization technique (Palmer 1985). This technique is used to determine the extent of overlap between any two clones and to determine the internal and border fragments (Heinhorst et al. 1988). Equation (5.38) $S = M_{xy}/[M_x - M_y]$ of Nei (1987) was used to calculate the proportion of shared restriction sites between the species.

Results

The restriction enzymes *Pst*I, *Pvu*II, *Sal*I, *Kpn*I and *Xho*I produced an average of 13 fragments per enzyme. Figure 1 illustrates the typical hybridization patterns obtained when radio-labelled barley cpDNA clones were hybridized to a Southern blot containing digested *Paspalum* DNA. The estimated molecular size, copy number and the heterologous probe(s) to which each of the digested fragments hybridized is listed in Table 1. Copy number was determined by the intensity of the hybridization patterns. In general, fragments that comprise parts of the inverted repeat (IR) region are represented twice and appear as very intense bands. The total chloroplast genome size of the *Paspalum* species was estimated to be approximately 139.3 kbp. The cpDNA physical maps are shown in Fig. 2. No mutational differences were observed between the cpDNAs after digestion with *Kpn*I. A total of eight restriction site mutations were detected with *Pst*I, *Pvu*II, *Sal*I and *Xho*I (Table 2). Most of these mutations occurred at the ends of the large single-copy (LSC) region. For example, in the *Pst*I digests, the P2/3 probes hybridized to a 19.3 kbp fragment in *P. notatum* and two fragments of 15.9 kbp and 3.4 kbp in *P. dilatatum* (Table 1). The sum of the latter two fragments was equivalent to 19.3 kbp. This information suggests that there was loss/gain of a restriction site. Fragments of

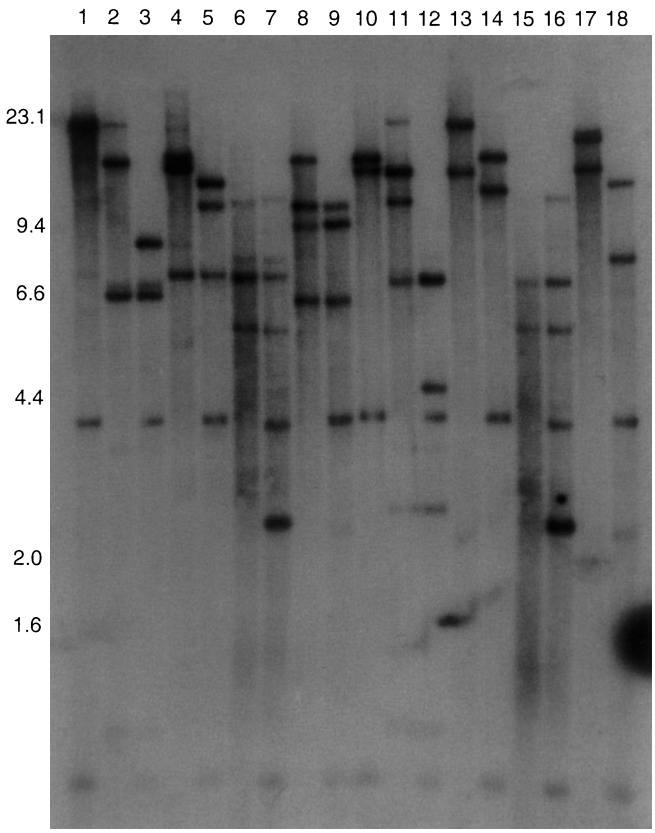

Fig. 1 Autoradiograph showing hybridization patterns of P3 barley cone to total DNA digests of *Paspalum*. The lanes represent DNA digested with *Pst*I (*1*), *Pvu*II (*2*), *Pst*I/*Pvu*II (*3*), *Sal*I (*4*), *Pst*I/*Sal*I (*5*), *Kpn*I (*6*), *Pst*I/*Kpn*I (*7*), *Xho*I (*8*) and *Pst*I/*Xho*I (*9*) for *P. notatum*. *Lanes 10–18* represent similar digests for *P. dilatatum*. The *numbers* in the left margin represent molecular-weight marker sizes in kbp

Table 1 *Paspalum* cpDNA fragment sizes, copy number and barley and wheat probes to which hybridization occurred

Enzyme	Fragment size		Copy number	Probe-fragment hybridization
	dilatatum	*notatum*		
*Pst*I	20.0	20.0		P5, P7
		19.9		P1, B(10-18)
		19.3		P2, P3, B(21-8)
		19.2		P2, P3, B(21-8)
	15.9			P2, P3, B(10-18), B(21-8)
	15.0			P1, B(10-18)
	14.5			P2, P3, B(21-8)
	14.3	14.3		P4, B(2-15)
	12.1	12.1		P6, P9
	5.8	5.8	3	P6, P8, P9, B(21-8)
	5.2	5.2		P10
	4.9			P1, B(10-18)
	4.7			P2, P3
	3.9	3.9		P3
	3.4	3.4	2	P8
	0.6	0.6	2	P2, P3

Table 1 Continued

Enzyme	Fragment size		Copy number	Probe-fragment hybridization
	dilatatum	*notatum*		
*Pvu*II	22.5	22.5		P1
	18.2	18.2		P6, P9, P10
		17.4		P2, P3, P8, B(10-18)
	16.3	16.3		P4, B(2-15)
	14.8			P2, P3, P8, B(10-18)
	14.7	14.7		P5, P7
	7.1	7.1		P4, P7, B(2-15)
	5.9	5.9		P2, P3
	4.5	4.5		P8
	4.0	4.0		P2
	3.4	3.4	2	P6, P8, P9
	2.6			P2, P3
*Sal*I	27.2	27.2		P2, P4, B(2-15)
	23.2	23.2		P6, P9, P10
	22.3			P2, P3, B(10-18), B(21-8)
		15.2		P2, P3, B(10-18) B(21-8)
	14.1	14.1		P2, P3
	12.7	12.7		P5, P7
	8.9	8.9		P1
	7.4	7.4	2	P8
	7.2	7.2		P1
		7.1		P3
	7.0	7.0		P1, P5
	1.3	1.3		P5
	0.6	0.6		P2
*Kpn*I				
		19.1		P2, P3
		18.9		P5, P7
		18.6		P6, P9, P10
		17.6		P1
		15.9		P4, B(2-15)
		11.2	2	P8
		7.9		P2, P3, B(10-18), B(21-8)
		7.1		P3
		5.7		P2, P3, B(10-18)
		2.1		P4, B(2-15)
		1.9		P6, P9
		0.8	2	P8
*Xho*I	34.4	34.4		P2, P4, P5, P7, B(2-15)
	20.3			P1, B(10-18)
	18.6			P2, P3, B(21-8)
		17.4		P8, P9, P10
	14.7	14.7	2	P2, P3, P8, P9, P10, (B21-8)
		12.2		P2, P3
		10.4		P1, B(10-18)
		9.9		P1, B(10-18)
	9.7	9.7		P5
		6.4		P3
	3.8	3.8		P1
	3.5	3.5		P8
	3.2	3.2	2	P6, P9
	2.8	2.8		P2
	2.7			P2
	2.4	2.4		P1
	0.9	0.9	2	P6, P9

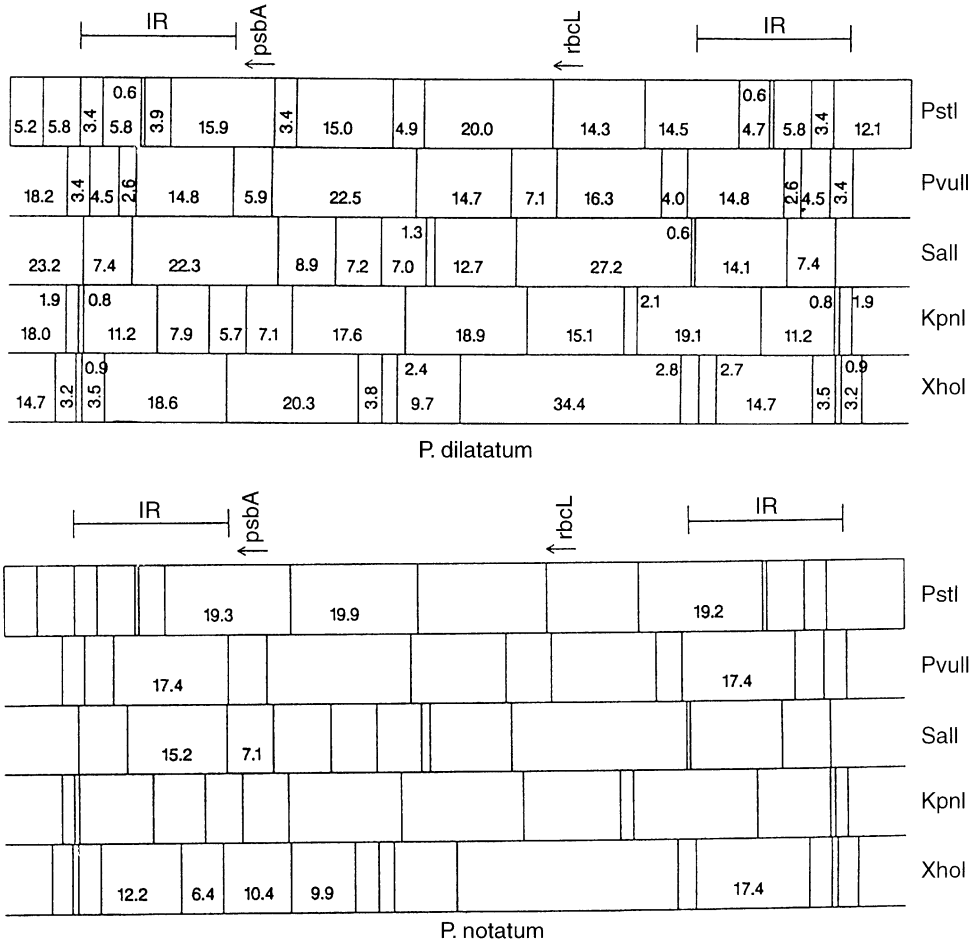

Fig. 2 Chloroplast DNA restriction site maps of *Paspalum dilataum* and *P. notatum*. Clevage sites for *Pst*I, *Pvu*II, *Kpn*I, *Sal*I and *Xho*I are shown. Fragment sizes are shown in detail for *P. dilatatum*. In *P. notatum*, only fragments that differ in size are indicated. The position of the inverted repeat (*IR*) regions are shown. Approximate locations of the *rbc*L and *psb*A genes are shown for orientation of maps. The maps are presented in linearized form starting at the center of the small single-copy region

Table 2 Restriction site mutations detected between the chloroplast genomes of *P. dilatatum* and *P. notatum*. Mutations were polarized by comparison with the outgroup species, *Panicum virgatum*. The first fragment listed for each mutation represents the primitive state

Enzyme	Probe	Change	Mutated species
*Pst*I	P1	15.0 + 4.9 = 19.9	*P. notatum*
*Pst*I	P2	14.5 + 4.7 = 19.2	*P. notatum*
*Pst*I	P2/P3	15.9 + 3.4 = 19.3	*P. notatum*
*Pvu*II	P2/P3	14.8 + 2.6 = 17.4	*P. notatum*
*sal*I	P3	22.3 = 15.2 + 7.1	*P. notatum*
*Xho*I	P1	20.3 = 10.4 + 9.9	*P. notatum*
*Xho*I	P2	14.7 + 2.7 = 17.4	*P. notatum*
*Xho*I	P3	18.6 = 12.2 + 6.4	*P. notatum*

approximately 15.9 and 3.4 kbp were also observed in the outgroup species. *Panicum virgatum*. This implies that the character state in *P. dilatatum* is plesiomorphic (primitive state). We have polarized the mutations listed in Table 2 by using *P. virgatum* as the outgroup. The proportion of shared restriction sites between the two species was estimated to be $S = 0.90 +/- 0.03$.

Discussion

The chloroplast genome represents a very conservative genetic system with an estimated rate of nucleotide substitutions to be approximately $0.3–0.8 \times 10^{-9}$ per site per year (Birky 1988). Despite this intrinsically low rate of change, the two *Paspalum* species have well-differentiated chloroplast genomes identifiable by eight restriction site mutations (Table 2, Fig. 2). This study showed that restriction site analysis of cpDNA provides useful species-specific markers for the identification of *P. dilatatum* and *P. notatum*. Restriction site variation in cpDNA probably arises from base pair mutations which create or eliminate a particular cleavage site. Most of the site changes appeared to be concentrated at both ends of the LSC region (Fig. 2). A similar phenomenon has been reported for *Nicotiana* (Yang et al. 1992). However, other studies (Ogihara and Tsunewaki 1988; Kellogg 1992) have shown that cpDNA restriction site variation is more common at the end of the LSC that contains most of the tRNA genes and the termini of the three grass-specific

inversions. The data from this study suggest that both ends of the LSC region are probably hotspots for cpDNA mutations. Further physical mapping studies from other plant groups are warranted to confirm our finding.

The distinct chloroplast genomes of the two species are congruent with the morphological and cytogenetical differences between them and their placement in two distinct taxa: the *Dilatata* (*P. dilatatum*) and *Notata* (*P. notatum*) groups. The distinguishing morphological features between *P. dilatatum* and *P. notatum* include (1) the mat-forming habit of *P. notatum* compared to the caespitose habit of *P. dilatatum* (2) the stout, exposed rhizomes of *P. notatum* and the thick, short rhizomes of *P. dilatatum*, (3) the racemose panicle of usually two racemes in *P. notatum* and 3–7 racemes in *P. dilatatum* and (4) the short (15–60 cm) culms of *P. notatum* and the tall (61–122 cm) seedstalks of *P. dilatatum* (Radford et al. 1974; Barnes et al. 1995). The genomic constitution of *P. dilatatum* is IIJJX with X being the unknown genome, while that of tetraploid *P. notatum* is NNNN (Burson 1983; Quarin et al. 1984). No chromosome pairing has been observed in hybrids from diploid *P. notatum* × *P. dilatatum*, suggesting that there is little or no homology between the genomes of these two species and that the genomes of the two species are distinctly different (Burson and Bennett 1972). If we assume that the two *Paspalum* species had a common origin, tremendous genomic differentation would appear to have occurred during divergence and speciation. It appears that changes in the nuclear genome were accompanied by concomitant changes in the chloroplast genome. Such a feature has also been noted in tobacco and maize (Kung et al. 1982; Timothy et al. 1979).

The chloroplast genome size of *Paspalum* was estimated to be about 139.3 kbp. This value is similar to those reported for other grass species in the subfamily *Panicoideae* including maize, foxtail millet, job's tears and sorghum, all with chloroplast genome sizes of approximately 140 kbp (Enomoto et al. 1985). This contrasts with the smaller cpDNA size of species in the subfamily Pooideae, which is reported to be about 135 kbp (Soreng et al. 1990). This suggests that the chloroplast genome has undergone distinct size changes in plants of different grass subfamilies. A possible mechanism for this size difference is the movement of DNA from the chloroplasts to other organelles. Plastid and nuclear DNA sequences have been found in the mitochondrial genomes of several species (Stern and Lonsdale 1982; Nugent and Palmer 1988; Watanabe et al. 1994). The overall structural organization of the *Paspalum* cpDNAs is rather similar to those of most cpDNAs from other plants. The basic features of the *Paspalum* cpDNA include a set of inverted repeats approximately 23 kbp in length, a small single-copy (SSC) region of 11 kbp and a LSC region of 83 kbp. The cpDNAs of most higher plants show a typical genome structure having IRs of 20–26 kbp, SSCs of 10–20 kbp and LSCs of 80–100 kbp (Palmer 1985). The *Paspalum* chloroplast genome is also colinear with those of barley and wheat and possibly with those of other higher plants. The gene order of the chloroplast genome is generally highly conserved among higher plants (Palmer et al. 1988).

The chloroplast genome is predominantly maternally inherited in flowering plants (Kirk and Tilney-Bassett 1978; Sears 1983). Therefore, cpDNA holds certain advantages for studying the origin and specific parentage of polyploid plants (Palmer 1987). Chloroplast DNA has been shown to play a role in establishing the origin of several polyploid crop plants including wheat, potato, tobacco and *Brassica* species (Palmer 1987). A survey of chromosome numbers (Quarin and Norrmann 1987; Honfi et al. 1990) suggests that polyploidy is widespread in the genus *Paspalum*. Apomixis, uneven ploidy numbers, irregular meiosis and low pollen viability in *P. dilatatum* (Burson 1991) are other reasons why *P. dilatatum* cannot be used in establishing its phylogeny. These constraints are circumvented by using cpDNA for phylogenetic interpretation. It is envisaged that a detailed investigation of cpDNA analysis in the genus *Paspalum* will be useful in identifying the exact parentage of *P. dilatatum* and *P. notatum*.

Chloroplast DNA has become an ideal molecular tool for the construction of phylogenetic relationships. We have examined cpDNA restriction patterns of a number of species of *Paspalum* (unpublished data). The chloroplast genome structure of the species obtained in this study will permit correct interpretation of a detailed analysis of rearrangements, inversions, insertions/deletions and restriction site polymorphisms in the genus *Paspalum*.

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