EVOLUTIONARY PATTERNS IN THE DILATATA GROUP (*Paspalum*, POACEAE): A POLYPLOID/AGAMIC COMPLEX

By

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by

Pablo R. Speranza

This dissertation is dedicated to my son Mauricio who never forgot to give me a hug when I left home for work at night.

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EVOLUTIONARY PATTERNS IN THE DILATATA GROUP
(*Paspalum*, POACEAE): A POLYPLOID/AGAMIC COMPLEX

By

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Chair: Pamela S. Soltis
Major Department: Botany

Paspalum dilatatum Poir. and its related species are warm-season grasses native to temperate South America. The members of the Dilatata group include polyploid sexual and apomictic components, some of which have reached worldwide distributions. The common biotype of *P. dilatatum* is a complex apomictic pentaploid hybrid, and efforts to identify its putative ancestors have led to the accumulation of a significant amount of cytogenetic information about the relationships among biotypes within the Dilatata group. In general, past work in this complex has suffered from the lack of representative collections, and the low power of the techniques. In this study, I developed microsatellite markers, analyzed their transferability within the Dilatata group, and applied them to representative samples to analyze the evolutionary relationships within the group at different levels. The markers developed here show great power to detect recent hybridization and analyze genetic structure. The genetic structure of the sexual biotypes was described for a collection of *P. dilatatum* ssp. *flavescens*. This biotype is highly

autogamous, and its genetic variability does not show significant geographical structure probably due to continuous disturbance of the roadside environment it inhabits. The relationships among the sexual and the apomictic components are analyzed, and the origin of the apomictic biotypes is discussed. Genetic recombination was not detected in the apomictic hexaploids and tetraploids. Among the pentaploids, a single clone and its somatic variants were found on all the continents and in almost all the collection sites in its native area. All the other apomicts in the group, including the recombinant pentaploids are hypothesized to be derived from the pentaploid form. The probable mechanisms involve either the production of unreduced female gametes or eu-triploid pollen grains by the pentaploids. This is probably the most extensive study ever attempted in this group, and it will undoubtedly change the direction of all future research in these species. The new recombinant forms will have to be analyzed and their productive potential assessed, while existing collections should be re-structured to reflect the unexpected distribution of the genetic variability they contain.

CHAPTER 1
AN INTRODUCTION TO THE STUDY OF THE DILATATA GROUP OF *Paspalum*

A Historical Perspective

The genus *Paspalum* contains ca. 350 species, most of them native to the tropical and warm temperate New World (Chase 1929). Chase (1929) recognized about 20 informal taxomomic groups within the genus based on vegetative and reproductive morphological characters, a classification that is still widely used.

The Dilatata group of *Paspalum* contains several species with great forage potential, and several of them have been used as forage crops (Skerman and Riveros 1992). *Paspalum dilatatum* Poir. and its related species are warm-season grasses native to the grasslands of temperate South America. Some members of the group, particularly *P. dilatatum* ssp. *dilatatum* and *P. urvillei* Steud., have reached worldwide distributions wherever a warm temperate climate combined with sufficient rainfall exists.

The members of the Dilatata group have been classified into several formal and informal taxonomic categories which will be referred to as biotypes in this dissertation. The common biotype of *P. dilatatum* (*P. dilatatum* ssp. *dilatatum*) is a complex apomictic pentaploid hybrid, and efforts to identify its putative ancestors have led, over several decades, to the accumulation of a significant body of cytogenetic information about the relationships among all the species and biotypes within the Dilatata group and between this group and the related Virgata group (Table 1-1). The fist comprehensive treatment of the Dilatata species was done by Moraes Fernandes et al. (1968) based solely on the meiotic behavior of the biotypes. Burson (1983) summarized the results of

the advancements achieved during the 1970s by several interspecific hybridizations and assigned the genomes in the group to putative diploid donors. Several new tetraploid members of the group have been identified since then, but no significant advances have been made about the relationships among them and the apomictic components.

Table 1-1. *Paspalum* species known to share genomes with *P. dilatatum* Poir

Species or biotype	2n	Genomic Formula	Authority
Paniculata Group			
P. paniculatum	20	JJ	Burson (1979)
P. jurgensii	20	JJ	Burson (1978)
Dilatata Group			
P. dilatatum ssp. *dilatatum*	50	IIJJX	Burson (1983)
P. dilatatum ssp. *flavescens*	40	IIJJ	Burson et al. (1973)
P. dasypleurum	40	IIJJ	Quarín and Capponio (1995)
P. urvillei	40	IIJJ	Burson (1979)
P. dilatatum Virasoro	40	IIJJ	Caponio and Quarín (1990)
P. dilatatum Vacaría	40	IIJJ	Quarín unpub. res.
P. dilatatum "Chirú"	60	IIJJXX	Burson (1991)a
P. dilatatum Uruguaiana	60	$IIJJX_1X_2$	Burson (1995)
Virgata Group			
P. conspersum	40	I_2I_2JJ	Burson (1978)
P. virgatum	40	IIJ_2J_2	Burson and Quarín (1982)
P. rufum	20	II	Quarín and Norrmann (1990)
Quadrifaria Group			
P. haumanii	20	II	Quarín and Norrmann (1990)
P. brunneum	20	II	Quarín and Norrmann (1990)
P. quadrifarium	20	II	Quarín and Norrmann (1990)
P. intermedium	20	II	Burson (1978)
P. densum	20	II	Caponio and Quarín (1993)
P. durifolium	60	$IIJ_2J_2X^*X^*$	Burson (1985)

* unknown genome not related to other X genomes

Several possible donors have been suggested for the I genome of the Dilatata group, most of which constitute species commonly included in the Quadrifaria group as defined by Barreto (1966). This group has been seen as including several species typically based on self-incompatible sexual diploids and their apomictic autopolyploid cytotypes. Recently, an analysis of the relationships among the proposed sources of the I genome was prepared in collaboration with M. Vaio. This study addresses the relationships among the several proposed donors of the I genome to the Dilatata group using two main approaches: the distribution of rDNA sites in the genomes and phylogenetic analysis of the chloroplast sequences.

Our results suggest that the relationships among the species of the group are complex, with several polyploids of interspecific origin. Remarkably, the pairing ability of the chromosomes among the I genome species (Quarín and Norrmann, 1990) bears a correlation with the phylogenetic distances among them inferred from chloroplast sequences.

The analysis published in Vaio et al. (2005) is part of a phylogenetic analysis of the genus as a whole that has been undertaken by G. H. Rua and myself to provide a framework in which the origin of polyploid species complexes of *Paspalum* can be analyzed. This study is not yet completed as we still lack cytogenetic information on most of the samples included in the study. A summary of the current progress of this phylogenetic effort is shown in Fig. 1-1, and an outline of the methodology is provided in Fig.1-2. Concerning the origin of the Dilatata group, the relationships obtained so far suggest that the definitive identification of the genomic sources for the group is far from being achieved: the currently proposed sources of I genomes form a paraphyletic assemblage within which the proposed J genome donor (*P. juergensii*/*P. paniculatum*) is also found. Moreover, *P. rufum*, whose chromosomes also show a moderate degree of pairing to the I genome, is located in a different clade which includes, among others, most members of the Notata and Plicatula groups. If these relationships are confirmed in the future, the J genome would represent a derived genome nested in a group in which the plesiomorphic condition is the ability to pair with the I genome. The current identifications of the I and J genomes are the result of the knowledge that was available to the researchers when findings were made. Without a general understanding of the

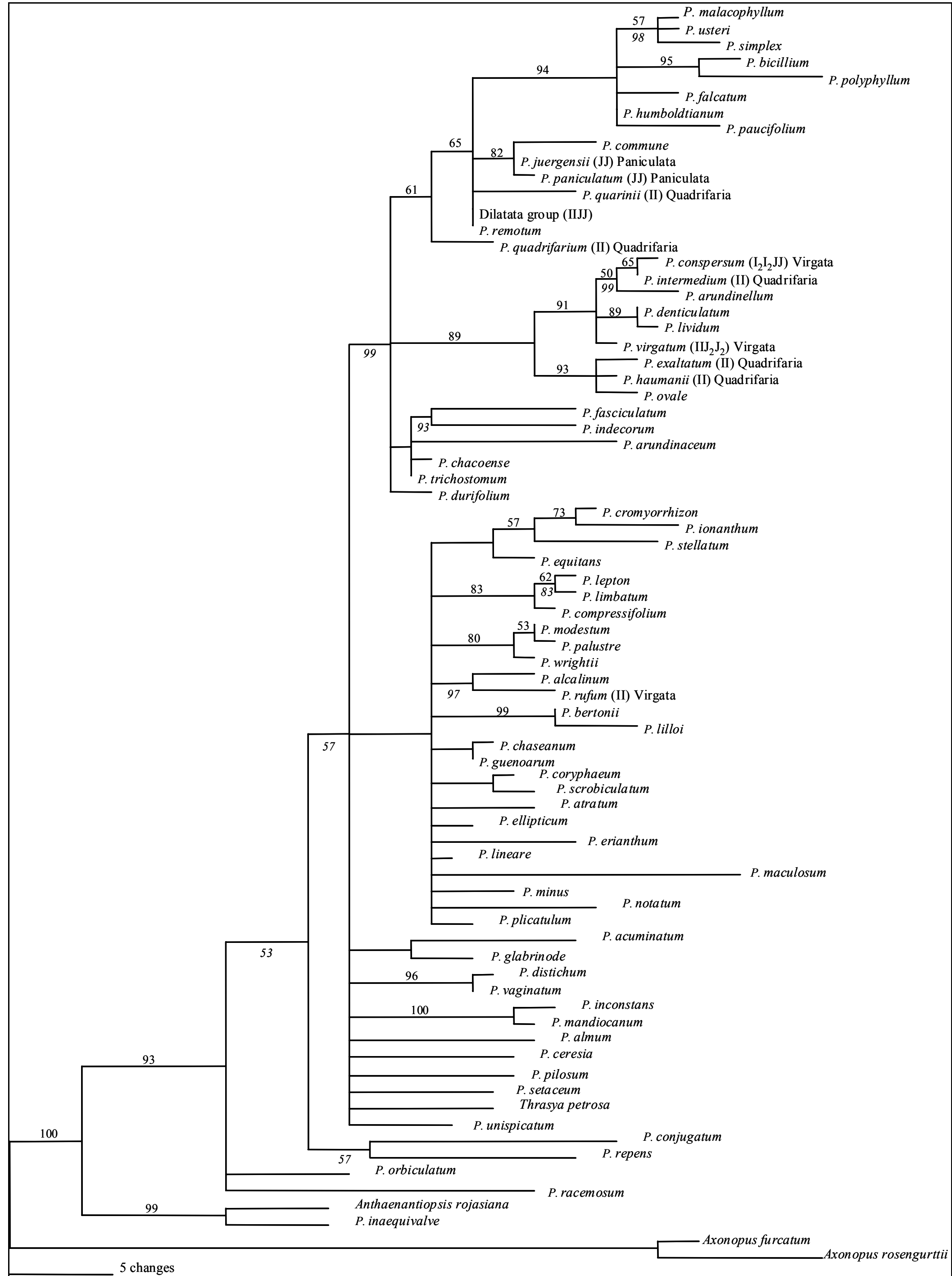

Fig. 1-1. Strict consensus of 15264 most parsimonious trees based on four cpDNA non-coding sequences (see Box 1). Numbers above branches represent boostrap support values, and numbers below the branches represent percent posterior probabilities for the same branches in a Bayesian (see Box 1) tree of similar topology when they were not 100%.

The phylogeny shown in Fig. 1-1 represents the current status of an ongoing project aimed at establishing a framework for the study of the relationships among species and polyploid complexes within the genus *Paspalum*. This project is being carried out in collaboration with G. H. Rua. The genus itself has not been analyzed using molecular tools, but some partial analyses show that the genus is not monophyletic if the species of *Thrasya* are excluded, and its nearest relatives are *Anthaenantiopsis*, *Thrasyopsis*, and some species currently assigned to *Panicum* (Gómez-Martínez and Culham 2000, Giussani et al. 2001, Duvall et al. 2001, Aliscioni et al. 2003).
The inclusion of multiple polyploids and hybrids in a phylogenetic analysis would confound the phylogenetic signal if we analyzed nuclear markers (including morphology). As a consequence we were faced with the practical impossibility of assembling a collection of only diploids or performing broad scale nuclear sequence isolation and cloning of a big portion of the genus, while the ploidy levels of most of our materials would be unknown. We decided to use a mostly living collection at the Universidad de Buenos Aires, for which cytogenetic information could be obtained and voucher specimens had been deposited. We undertook the phylogenetic effort using chloroplast sequences. With this kind of marker, a congruent set of sequences could be obtained, and our phylogenetic tree would represent the organismal history of our diploid samples and the maternal progenitors of our polyploids. This approach would circumvent the risk of insufficient taxon sampling by filling the gaps created by unrepresented diploids with the chloroplast sequences of their derived polyploids. Valuable information can also be obtained on the maternal origins of the polyploids. Four non-coding chloroplast regions were amplified: the *trn*L(UAA) intron, the *atpβ-rbcL* spacer, the *trn*G(UCC) intron and the *trn*L(UAA)-*trn*F(GAA) spacer (technical details on amplification and sequencing are given in Vaio et al. 2005). A single matrix was made of all the alignments for 72 species of *Paspalum*, one species of both *Thrasya* and *Anthaenantiopsis*, and two species of *Axonopus* as outgroups. The matrix was analyzed with parsimony using PAUP* (fully heuristic search with 20000 SAR) and Bayesian approaches using MrBayes (a model was selected with Modeltest and the MCMC was run for 2,000,000 generations on MrBayes). The trees obtained with both approaches, were fully congruent, and the tree obtained with MrBayes was nearly identical to the majority-rule consensus obtained with parsimony. A slightly more conservative parsimony strict consensus is shown in Fig. 1-1 which does not show nine nodes that appeared in the Bayesian tree with posterior probabilities mostly below 0.70.

Fig 1-2. Main conclusions of a preliminary phylogeny of the genus *Paspalum*

.

In spite of the data being preliminary, a few well-supported hypotheses can be derived from this tree concerning the placement and origin of the Dilatata group:

1. *Paspalum* is not monophyletic unless *Thrasya* is included in it and a) *Anthaenantiopsis* is included or b) *P. inaequivalve* is excluded.

2. *Paspalum racemosum*, *P. orbiculatum*, *P. repens,* and *P. conjugatum* form a basal grade to an otherwise poorly supported clade containing the rest of the species of *Paspalum* and *Thrasya*.

3. Two major clades with high posterior probabilities but no parsimony bootstrap support include most of the species analyzed.

4. The species of the Dilatata, Virgata, Paniculata, and Quadrifaria groups are included in the same clade but in different subclades. The species of the Quadrifaria group are found in both subclades.

5. The second major clade includes species of the Plicatula, Notata and Bertoniana groups among others. This clade shows very little internal resolution. *P. rufum* is included in this clade.

6. The chloroplast genome of the Dilatata group is included in a clade that includes species that have been assigned both the I and J genomes.

7. The chloroplast genomes of the Virgata group are scattered within the clade that contains most of the Quadrifaria species.

Fig. 1-2 (continued)

relationships among the main clades within the genus, the quest for the direct genome donors of the polyploid groups (including the Dilatata group) is deemed to continue at random, with occasional successes and many false positives.

The Contributions of This Dissertation

In general, past work in this complex has suffered from two main limitations: the lack of representative collections, and the low power of the techniques. An effort was made in this case to represent with more than one individual each component of the complex. The main source of materials was a collection deposited by myself between 1992 and 1999 in the Germplasm Bank at the Facultad de Agronomía, Montevideo, Uruguay, which was complemented with the USDA collection.

The main body of this dissertation is divided into four chapters dealing with different levels of analysis of the Dilatata complex.

Chapter 2: The Technique

Two recent works (Speranza et al. 2003, Vaio et al. 2005) represent the first examples of the use of cytogenetic information in this group beyond the sheer number of chromosomes or their meitotic behavior; however, cytogenetics alone cannot answer many of the questions that must be addressed in this group. It has been hypothesized that, for example, the pentaploid biotype may be the direct derivative of Chirú and a sexual tetraploid. Microsatellites, due to their co-dominant nature and high degree of variability, appear as the ideal kind of markers to test such hypotheses. Furthermore, other issues that need to be addressed, such as the breeding systems and genetic structure of several biotypes, could also be effectively resolved using variable co-dominant markers.

In Chapter 2, I will design and characterize several microsatellite loci for *P. dilatatum* ssp. *flavescens* and test their transferability to all the other members of the Dilatata group. I will also attempt to predict their potential to test different types of

hypotheses. These markers will be used as the main source of information in the following three chapters.

Chapter 3: The Germplasm

In the context of the traditional hypothesis, *P. dilatatum* is the product of a tetraploid (IIJJ) x hexaploid (IIJJXX) hybridization. The use of a variable, well-characterized collection of tetraploids seems to be the most direct resynthesis route for variable pentaploids. This collection could be hybridized to either of the two apomictic and invariable hexaploids to produce new pentaploids. In spite of this, representative collections of tetraploids have not been available. The collection of *P. dilatatum* ssp. *flavescens* that I used here was the first one made with such an objective in mind, and a molecular characterization of it will greatly increase its value. A comprehensive populational-level study of the genetic variability and its structure will be presented in Chapter 3.

Chapter 4: The Complex

Two previous attempts have been made to represent the Dilatata group in its entirety. These attempts (Moraes Fernandes et al. 1968) and Burson (1983) were based on the knowledge available at the time. In spite of the identification of new components and new relationships mainly during the 1990s, the paucity of information, the lack of comprehensive collections, or the limited interpretability of the markers used (see Casa et al. 2002), no great advances have been made in the past two decades. With the power of suitable techniques and a sufficient collection, Chapter 4 will surely become a landmark in the understanding of the complex.

Chapter 5: The All-Important Pentaploid(s)

It was the interest in this biotype that initiated a long series of studies in the Dilatata group. Since the establishment of its complex hybrid origin (Bashaw and Forbes 1958), the biotype has mostly been seen as a static, invariable entity. Chapter 5 is probably the most extensive and intensive study of genetic variability ever attempted in this biotype, and will undoubtedly change the direction of all future research on it

CHAPTER 2
NUCLEAR AND CYTOPLASMIC MICROSATELLITE MARKERS FOR THE SPECIES OF THE DILATATA GROUP OF *Paspalum* (POACEAE)

Introduction

The Dilatata group of *Paspalum* is a polyploid complex native to the grasslands of temperate South America. The complex contains several informal taxonomic entities that will be generally referred to as biotypes in this paper. *Paspalum dilatatum* Poir. ssp. *dilatatum*, a trihybrid pentaploid apomict (Bashaw and Forbes 1958, Bashaw and Holt 1958), is a widely recognized forage crop. This biotype has been assigned the IIJJX genomic formula (Burson 1983). The Dilatata group also includes several sexual selfing allotetraploids and several tetra- and hexaploid apomictic entities. The sexual allotetraploids (*P. urvillei* Steud., *P. dasypleurum* Kunze ex Desv., *P. dilatatum* ssp. *flavescens* Roseng. Arr. et Izag., and biotypes Virasoro and Vacaria of *P. dilatatum*) have been shown either directly or indirectly to share the IIJJ genomic formula (Burson et al. 1973, Quarín and Caponio 1995, Burson 1979, Caponio and Quarín 1990), and their interfertility has been either directly assessed by hybridization (Caponio and Quarín 1990, Quarín and Caponio 1995) or inferred from the occurrence of natural hybrids (Valls and Pozzobon 1987). The group also includes two apomictic hexaploids (*P. dilatatum* biotypes Uruguaiana and Chirú) which have been assigned the $IIJJXX_2$ and IIJJXX genomic formulae, respectively (Burson 1991, 1992). *Paspalum dilatatum* Torres, an asynaptic apomictic hexaploid (Moraes-Fernandes et al. 1968), and *P. pauciciliatum*, an apomictic tetraploid (Bashaw and Forbes 1958) of unknown genomic

constitution, are also included in the group. Recently, Machado et al. (2005) have shown that there may be several pentaploid apomictic entities in the group which have not yet been described or named.

Interest in breeding the common pentaploid biotype has been the main motivation for extensive interspecific hybridization and cytogenetic analyses in this group. Pentaploid (IIJJX) resynthesis by hexaploid (IIJJXX) x tetraploid (IIJJ) hybridization has been suggested as a possible breeding strategy for the group (Burson 1983). Vigorous synthetic pentaploids have been successfully obtained by this kind of cross (Burson 1991b, 1992, Speranza 1994, unpubl. res.); however, the evaluation of available genetic variability, particularly in the selfing tetraploids, has not been undertaken.

Sufficient knowledge has been accumulated not only to initiate the first breeding attempts, but also to make this species complex an interesting model for the study of apomixis and polyploidy. In spite of this, the study of the relationships among the different entities that comprise this group has not advanced much in the last decade, most probably due to the limitations of the tools available and the biological characteristics of the organisms themselves. Only recently has it been possible to obtain further cytological information on these biotypes through the modification of cytological techniques that allow chromosome identification and karyotyping (Speranza et al. 2003, Vaio et al. 2005); however, cytogenetics alone cannot answer many of the questions that must be addressed in this group.

As most of the apomicts in the complex have been hypothesized to be inter-biotypic combinations, the markers required to address questions about their genetic structure and origin must be transferable among all of the putative parents involved and

preferably co-dominant. On the other hand, assessment of parentage would be best achieved with markers that are stable within biotypes, while the study of the genetic structure of the sexual components of the complex requires high levels of variability.

Attempts have been made to use molecular markers for the study of these species, but even when some degree of genetic differentiation between the biotypes and intra-biotypic variability were confirmed (isozymes: Pereira et al. 2000, Chies unpublished data, Hickenbick et al. 1992, AFLP: Speranza unpublished data, Casa et al. 2002), the levels of variability have not been high enough or their interpretability has been very limited. Allopolyploidy adds an additional level of complexity to the genetic interpretation of molecular markers: the assessment of homology vs. homeology among markers may not be straightforward, particularly in complex interbiotypic combinations. Microsatellite markers, despite the greater technological investment required for their development, provide the best tools for the study of the issues that have to be addressed: genetic structure, relatedness among the entities of the complex, and parentage of the apomictic components which are expected to be fixed hybrids. The generalized use of simple sequence repeat (SSR) enrichment and PCR-based protocols has greatly facilitated the development of new microsatellite loci (Fischer and Bachmann 1998, Kijas et al. 1994, Kandpal et al. 1994, Jakše and Javornik, 2001). Microsatellites not only provide more powerful genetic data due to their co-dominant nature, they usually tend to be extremely variable. Mutation rates of nearly $1x10^{-3}$ have been directly observed in maize (Vigouroux et al. 2002). In well-studied selfing grass amphiploid systems like wheat, microsatellites are capable of revealing great genetic variability (Röder et al. 1995) where

isozyme, RFLP, and AFLP markers show a high degree of marker conservation (Hazen et al. 2002, Kim and Ward 2000).

Finally, determining the directionality of hybridizations within the species complex may be crucial to understanding the mechanisms by which new genetic combinations are being generated. Chloroplast microsatellites, typically $(T/A)_n$, have been successfully used to elucidate directional formation of allopolyploids in grasses (Ishii and McCouch 2000). Ishii and McCouch (2000) reported that despite successful cross-amplification, the presence of variable $(T/A)_n$ tracts was not conserved among distantly related grass genera; however, putatively universal primers for grass chloroplast microsatellites have been reported in the literature (Provan et al. 2004).

Sixteen variable nuclear and one variable chloroplast microsatellite loci for *P. dilatatum* ssp. *flavescens* were developed and characterized in this study. Their transferability among all the taxonomic entities of the Dilatata group was assessed, and their utility for addressing populational and phylogenetic studies at different levels is discussed.

Materials and Methods

Microsatellite Capture

A genomic DNA library consisting of Sau3AI fragments of *P. dilatatum* ssp. *flavescens* was enriched for putative microsatellite-containing sequences following the procedures of Ernst et al. (2004) with minor modifications. Briefly, genomic DNA was extracted with Sigma Genelute™ kit (Sigma-Aldrich, St. Louis, MO) and digested with Sau3AI. Fragments smaller than approximately 400bp were removed by fractioning using Chroma Spin® columns (Clontech Laboratories). Sau3AI linkers were ligated to the remaining fragments which were then amplified by PCR. The amplified fragment library

was enriched for $(GT)_n$-containing sequences by binding to a Vectrex® Avidin D matrix (Vector Laboratories, Burlingame, CA) to which a biotinylated $(CA)_n$ oligonucleotide probe had been previously bound. The eluted fragments were reamplified by PCR using primers for the Sau3AI linkers, ligated into pCR® II-TOPO ® plasmids (Invitrogen, Inc) and transformed into ONE shot ® *E. coli* competent cells. Colonies were screened by binding them to Magnacharge nylon transfer membranes (Osmonics, Inc.). The membranes were probed with labeled $(CA)_n$ and positive colonies detected with Lumi-Phos 480 (Lifecodes, Inc.). All probe labeling, hybridization, and detection was carried out with Quick-Light TM system (Lifecodes, Stamford, CT). Positive colonies were grown overnight in a liquid medium and plasmids purified with QIAprep Spin Miniprep Kit (Qiagen, Inc.). Plasmid isolates were screened a second time by dot-blotting serial dilutions on nylon membranes and hybridizing to a $(CA)_n$ probe. Isolates showing consistent hybridization signal through the dilutions were sequenced and used for primer development.

Plasmid isolates were sequenced on a CEQ 8000 capillary sequencer (Beckman-Coulter, Fullerton, CA) using ¼ reaction volumes with the addition of 80mM Tris and 2 mM $MgCl_2$ (pH 9) to complete the volume of a full reaction. The sequences were edited manually using Sequencher^{TM} (V4.1.4, Genecodes, Ann Arbor, MI).

Primers were designed for sequences containing repeats longer than $(GT)_{10}$ with Primer 3 (Rozen and Skaletsky, 2000). Low-complexity regions were excluded for primer design when possible.

Chloroplast Markers

Sequences for 6 chloroplast non-coding regions were obtained for all the recognized entities in the Dilatata group (Table 2-1). Six regions were analyzed: the

trnT(UGU)-*trnL*(UAA) spacer, the *trnL*(UAA) intron, the *PsbA*-*trnH* spacer, the *atpB*-*rbcL* spacer, the *trnG*(UCC) intron, and the *trnL*(UAA)-*trnF*(GAA) spacer. PCR and sequencing conditions and primers were reported in Vaio et al. (2005) except for the *trnT*(UGU)-*trnL*(UAA) spacer which was amplified and sequenced using primers A and B (Taberlet 1991). Primers were designed flanking two poly-A repeats located in the *trnT*(UGU)-*trnL*(UAA) spacer. All primers reported by Provan et al. (2004) were also tested. A second poly A-tract not reported by Provan et al. (2004) was detected near the *trnL*(UAA)3' exon but no length variability was observed among the available sequences, and no further analysis was performed on it. Primer design, labeling, amplification, and detection procedures were performed as for the nuclear SSR described above.

Plant Material

Potential variability of microsatellites was assessed by analyzing a total of 28 accessions representing different species of the Dilatata group. To assess intraspecific variability, we analyzed ten accessions each of *P. dilatatum* ssp. *flavescens* and *P. urvillei*. Accessions were chosen to represent as much of the native range of the species as possible. To assess transferability, two accessions each of *P. dasypleurum*, *P. dilatatum* ssp. *dilatatum*, and biotypes Virasoro and Vacaria of *P. dilatatum* were also analyzed. Seed samples were obtained from the Southern Regional Plant Introduction station, Griffin, GA, USA and the Germplasm Bank at the Facultad de Agronomía, Montevideo, Uruguay. Collection localities and accession number information for all materials are shown in Table 2-1.

DNA was also extracted from 43 F_2 individuals derived from a hybrid between *P. dilatatum* ssp. *flavescens* and *P. dilatatum* Virasoro to analyze segregation patterns of loci and possible linkage relationships. Deviation from expected segregation ratios and

linkage between loci were assessed using Joinmap 3.0 (Van Ooijen and Voorrips, 2001). When there were indications that more than one locus had been amplified by a primer pair (see below), the loci were considered as putative homeologs and the absence of linkage between them regarded as a test for their homeology.

Table 2-1. Accession numbers and collection locations of the plant material used to test microsatellite transferability among biotypes

Species or biotype	Individual	Accession number*	Location
P. dilatatum ssp. *flavescens*	1	PI 508720	Pila, Buenos Aires, Argentina
2n=4x=40 (IIJJ)	2	PI 508722	Route 41, 1.0 km W of General Belgrano, Buenos Aires, Argentina
	3	N.A.7355	Route 3 near Trinidad, Florida, Uruguay
	4	N.A.7363	Route 8 km 34.3, Canelones, Uruguay
	5	N.A.7434	Riachuelo, Colonia, Uruguay
	6	N.A.7439	Route 12, 600 m N of Route 9, Maldonado, Uruguay
	7	N.A.7468	Route 56 km 51.500, Florida, Uruguay
	8	N.A.7470	Route 11 km 65.600, San José, Uruguay
	9	N.A.7476	Route 9 km 227, Rocha, Uruguay
	10	N.A.7492	Route 6 km 189.600, Florida, Uruguay
P. urvillei	1	PI 509008	Ivoti, Cascata de São Miguel, about 45 km N of Porto Alegre, Rio Grande do Sul, Brazil
2n=4x=40 (IIJJ)	2	PI 509010	Route BR 116, 6 km N of Pelotas River, Santa Catarina, Brazil
	3	PI 509012	Route 8, 162 km W of Buenos Aires, Buenos Aires, Argentina
	4	PI 509013	Villa Nueva, 3 km S of Villa Maria, Córdoba, Argentina
	5	PI 164065	Florianopolis, Santa Catarina, Brazil
	6	N.A.2957	Route 26, km 25, Paysandú, Uruguay
	7	N.A.7392	Road to Las Cumbres de La Ballena, Maldonado, Uruguay
	8	N.A.7389	Route 7, km 103, Florida, Uruguay
	9	N.A.7390	Route 29 near Minas de Corrales, Rivera, Uruguay
	10	N.A.7199	Balneario Solís, Maldonado, Uruguay
P. dilatatum Virasoro	1	N.A.7207	Gobernador Virasoro, Corrientes, Argentina
2n=4x=40 (IIJJ)	2		Garruchos, Corrientes, Argentina. Voucher: BAA24352
P. dilatatum Vacaria	1	PI 404370	Near Vacaria, 192 km on Route BR 116, N of Porto Alegre, Rio Grande do Sul, Brazil
2n=4x=40 (IIJJ)	2	PI 404382	On Route BR 285, 10 km west of Vacaria, Rio Grande do Sul, Brazil
*P. dasypleurum***	1		Botanical Garden, Valdivia, Chile
2n=4x=40 (IIJJ)	2		General Lagos, Valdivia, Chile
P. dilatatum ssp. *dilatatum*	1	N.A.7542	Quebrada de los Cuervos, Treinta y Tres, Uruguay
2n=5x=50 (IIJJX)	2	N.A.7673	Masoller, Rivera, Uruguay

*Accession numbers preceded by PI correspond to the Southern Plant Introduction Station, Griffin, GA, USA. Numbers preceded by N.A. correspond to the Germplasm Bank at the Facultad de Agronomía, Montevideo, Uruguay.
** Seeds of *P. dasypleurum* were kindly provided by Ing. For. Rodrigo Vergara from the Universidad Austral de Chile and the University of Florida.

F_1 hybrids were obtained by manually emasculating a plant of *P. dilatatum* ssp. *flavescens* and pollinating it with pollen from an individual of *P. dilatatum* Virasoro.

Emasculation and pollination were carried out about one hour after sunrise. The plants to be emasculated were placed at approximately 20ºC and 100% RH to delay anther dehiscense after anthesis was initiated each morning. Mature full seeds were counted and germinated in Petri dishes on filter paper in an incubator with alternating temperatures (16 h at 30ºC light, 8 h at 20ºC dark). Germinators were placed at 4ºC for 4 days prior to incubation to break dormancy and homogenize germination. The resulting progeny were grown and the hybrids were identified by the high number of nerves in glume II and lemma I which characterize the pollen donor. Selfed seed of one F_1 hybrid was collected and treated as described above to establish the segregating F_2 progeny used in this study.

Amplification and Scoring

For all plant materials DNA was extracted from fresh leaves or silica-gel-dried leaves using Sigma Genelute™ kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

Amplification, labeling, and separation conditions were adjusted for all primer pairs following Boutin-Garnache (2001). Forward primers were extended by adding an M13 tail (5'-CACGACGTTGTAAAAC-3'), and M13 primers were labeled with D4 (Beckman Coulter, Fullerton, CA). All PCR amplifications were carried out in 10 µL reactions containing 0.2 units of NEB Taq polymerase (New England Biolabs, Beverly, MA), 1.5 mM $MgCl_2$, 0.15 µM of the reverse primer and labeled M13 primer, 0.01 µM of the extended forward 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 two-step program for all primer pairs. The PCR profile consisted of an initial denaturing step of 5 min at 94ºC, followed by 40 cycles of 15 sec at 94ºC and 3 min at 53ºC, and a final extension step of 5 min at 72ºC. Labeled microsatellite products were separated in a

CEQ 8000 capillary sequencer (Beckman-Coulter, Fullerton, CA) by loading 0.75 µL of the PCR product and 0.35 µL of CEQTM DNA Size Standard Kit-400 (Beckman Coulter, Fullerton, CA) in 25µL of formamide per well. Chromatograms were visualized on CEQ TM Genetic Analysis system software (Beckman Coulter, Fullerton, CA) and scored manually.

Alleles in different size ranges from different biotypes or subspecies were sequenced to assess homology. For primer pairs amplifying a single locus, alleles were amplified and sequenced directly from the PCR products of homozygous individuals. For primer pairs amplifying more than one putative locus, PCR products were separated in 2% agarose, and the bands were cut, purified with Wizard® SV Gel and PCR Clean-up System (Promega, Maddison, WI), and sequenced directly. When multiple bands could not be separated in the gel, gel slices containing several bands were cut and combined products cloned with a TOPO TA Cloning ® kit.

Results

Capture and Amplification Success

A total of 24 clones containing $(GT)_n$ repeats were captured and sequenced. Four clones were redundant. Primer pairs were designed for all clones, and all loci were amplified with the same two-step PCR profile detailed above. Fifteen of these primer pairs successfully amplified interpretable band patterns, and twelve of them were selected for further analysis based on a preliminary assessment of amplification success under the given conditions. Primer pairs for loci Pdfl1, Pdfl2, and Pdfl26 are reported but not further characterized (Table 2-2). Primer sequences, flanking sequence lengths, and repeat units for all successfully amplified loci are shown in Table 2-2.

Table 2-2. Primer sequences and structure for all the microsatellite loci reported in this study. When sequence information is available for more than one allele, the variable repeat motif is reported. GenBank accession numbers for the originally cloned sequence used to design each primer pair are given in parentheses.

Primer pair or locus	Primer sequences (5'-3')	flanking sequence length (bp)	Repeat unit
Focal loci			
Pdfl1 (DQ110403)	F-GGGCGTGACAAGATTGAGAG R-GATCCAACTCCTGGGATCAA	157	$(TG)_{26}C(GT)_5$
Pdfl2 (DQ110403)	F-GTCTTCTACGCGACAATGTA R-AAATGGTGGACGACACCTCT	170	$(AT)_5(GT)_{31}$
Pdfl4 (DQ110403)	F-TGGCTCATGTCAACCATGTC R-CTGGAGACCAAGCAAACAGG	161	$(TG)_{16}$
Pdfl6a (DQ110403)	F-GGTCCATCCTGCTGATGAAG R-AGCAGCACAACCTGCTGAG	167	$(GT)_{37}$
Pdfl7 (DQ110403)	F-TAGGCTGCGGAATCAACTTT R-ACAAGGACAAACCGACTGCT	189	$(GT)_{21}$
Pdfl8 (DQ110403)	F-AGGCTGCAGAAGACTCCAAA R-GCCACCTACTCCCCTCTGTA	182	$(GT)_{17}$
Pdfl10 (DQ110403)	F-GCTCATCAAATATGACTGAACCA R-TCTTACGTCCCACCCAAATC	142	$(TG)_8CG(TG)_{21}$
Pdfl11 (DQ110403)	F-AAGAAGCCATTGGGTCTGG R-CATGCATGCCTACACACAGA	142	$(TG)_{12}$
Pdfl12a (DQ110403)	F-TTCCTTTGTCAGTTCACTTCCAT R-ACAAACTGTGCGACAAGTGC	155	$(TA)_2$ $(GT)_{26}$
Pdfl15a (DQ110403)	F-AACCACTGTGTGAAGCTTGCTA R-TGTGCACACTCATCGAAAGA	152	$(GT)_2GC(GT)_{43}$
Pdfl18 (DQ110403)	F-GGAAGGTTCAGCAACGGATA R-GATAAGGCGGAGGGCTACTT	196	$(GT)_{12}$
Pdfl20a (DQ110403)	F-CTGGCCACTTCTTTGGACAT R-CGGCACTAGTTGCCTGAAA	162	$(TA)_8(TG)_{20}$
Pdfl22a (DQ110403)	F-GCATGCTGTTGTCTTTTGCT R-TTCCCTCGCCTCTGCTAGT	137	$(CT)_2(GT)_{30}$
Pdfl26 (DQ110403)	F-ATCGGCATGCTACAAGTTCC R-TCTCATGTTCATTGCTGAAGTG	99	$(CT)_{20}GC(GT)_{32}$
Pdfl28a (DQ110403)	F-AAAATACCCGTGCGTTGCTA R-CCACGCCATGTCGTCTACTA	159	$(TG)_{32}$
Non-focal loci			
Pdfl6b		148	$(TG)_2$
Pdfl12b		158	$(TA)_2$ AT $(GT)_6(GA)_8$
Pdfl15b		176	$(TA)_4$ $(GT)_6$
Pdfl20b		173	$(T)_{10}$
Pdfl20-3		151	$(TA)_6$
Pdfl22b		136	$(CT)_4$ $(GT)_{11}$
Pdfl28b		147	$(TG)_2$
Chloroplast locus			
cpDilB (DQ104323)	F-GGGAATCCGTAAAATGTCAGA R-GAAAAATTGATTTGCGAATTAGAGA	191	$(T)_{11}$

Primer pairs Pdfl6, Pdfl12, Pdfl15, Pdfl20, and Pdfl22 amplified more than one band in all tetraploid individuals, most of which appeared homozygous for all the other loci (Fig 1b, g, h, j, and k). In these cases, representative bands from both size ranges were cloned, sequenced, and compared with the originally captured sequences (Fig. 2-2). Two of the extra bands, a Pdfl12 190-bp band in *P. dilatatum* ssp. *flavescens* and a 140-

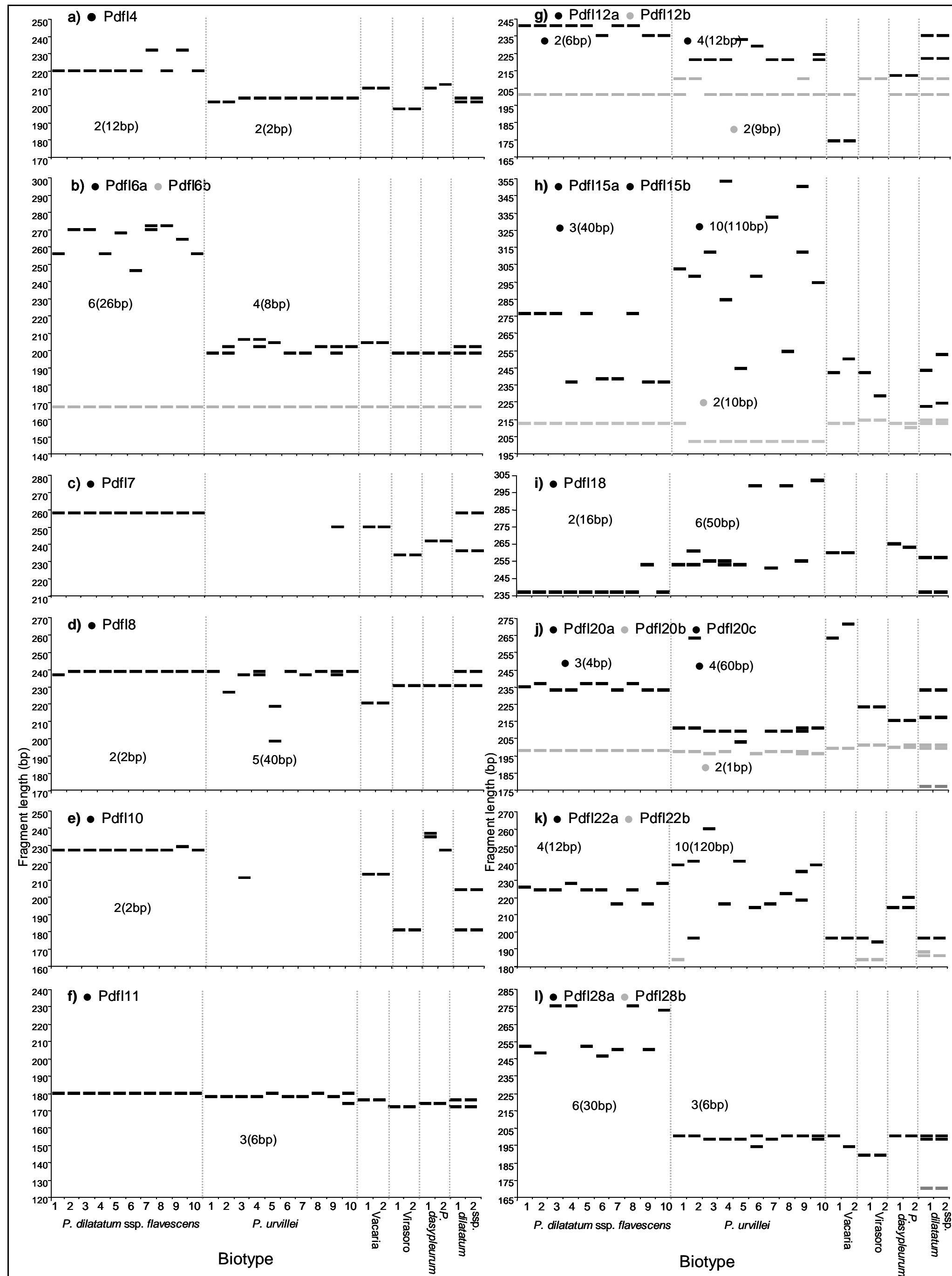

Fig. 2-1. A graphical representation of all the microsatellite alleles amplified for a sample of each biotype. Fragment lengths are plotted as scored including the M13 primer extension (19bp). Number of alleles within biotype are given for *P. dilatatum* ssp. *dilatatum* and *P. urvillei* for those loci that showed variability within biotypes. The corresponding maximum fragment size difference is shown in parenthesis

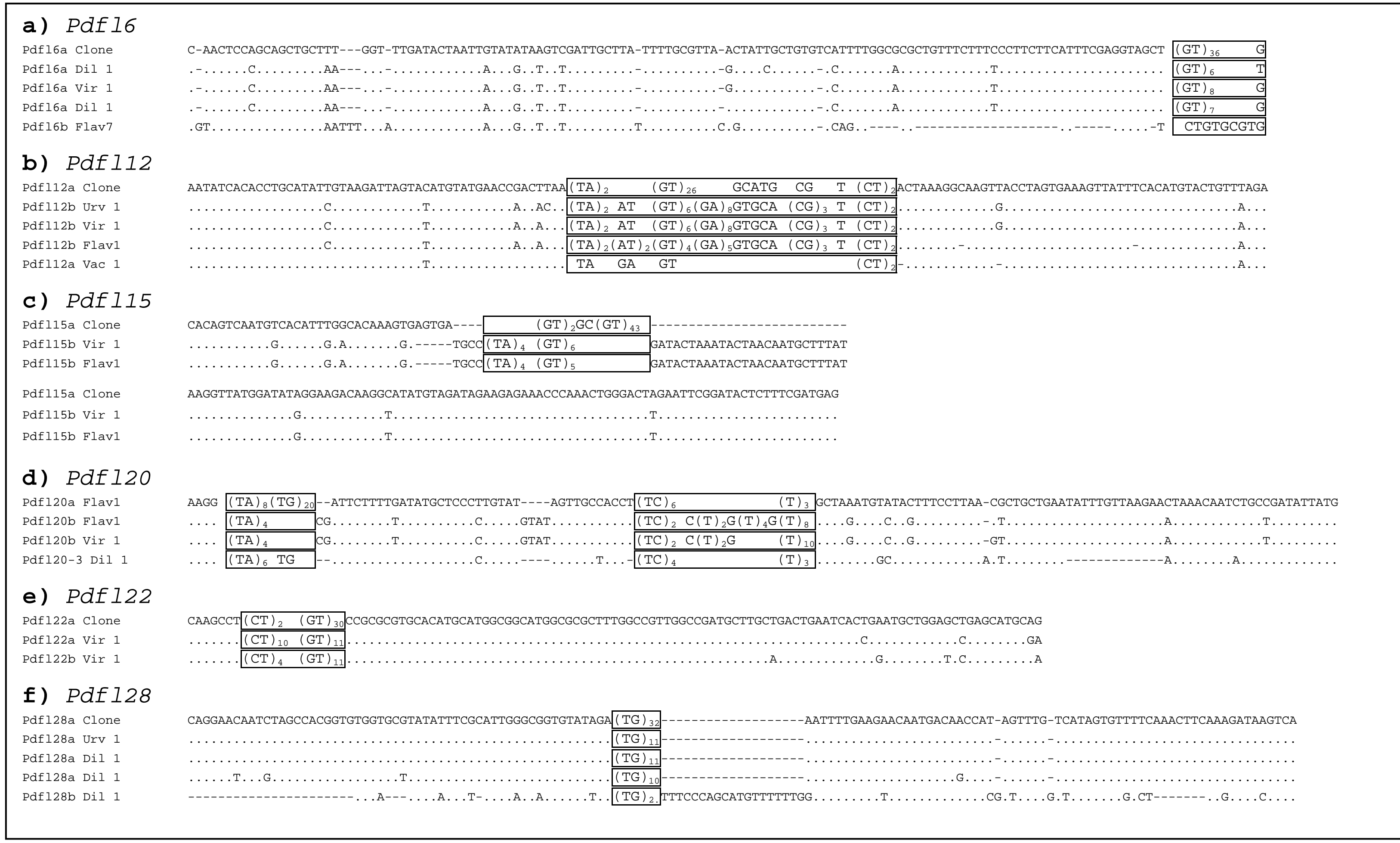

Fig. 2-2. Alignment of nucleotide sequences of representative alleles for the non-focal loci compared to the originally cloned sequences. Primer sequences are not included. All putatively homologous variable and non-variable repetitive sequences near or around the main microsatellite are highlighted for comparison

bp band in *P. urvillei* did not contain the forward primer sequence. These bands were in fact reproduced by using just the labeled M13 primer and the reverse primer for each locus. These bands were not further scored and are not shown in Fig. 2-2. In all the other cases, the extra bands showed extensive sequence similarity to the captured alleles (Fig. 2-2). The loci for which the primers were originally designed will be referred to as the focal loci. When two different loci were detected, the focal locus was identified by adding "a" to the primer pair name. For most non-focal loci, fragment sizes were smaller than the captured bands, and the microsatellite repeat was either absent or showed a lower number of repeats. The only exception was the Pdfl12a 140-bp allele in Vacaria which was smaller than the non-focal locus Pdfl12b. Representative allele sequences are shown in Fig. 2-2. For Pdfl20 and Pdfl28, an extra band was present in the apomictic pentaploid relative to tetraploids (Fig. 2-2j and l). These bands were sequenced, and they showed again extensive similarity to the captured loci (Fig. 2-2).

When more than one locus was amplified by a primer pair, alleles were assigned to either putative homeologous locus based on the sequences shown in Fig. 2-2. Homeologous loci are expected to be completely unlinked because they necessarily lie on different chromosomes. Linkage analysis based on the segregating F_2 population showed no evidence of linkage between loci amplified by the same primer pair with recombination frequencies ranging from 0.3664 to 0.4371 and LOD scores from 0.03 to 1.09 (significance threshold LOD equal to 3) (Table 2-3).

In addition, no significant linkage was observed when considering the full set of 16 loci (including non-focal loci), even at a low LOD score threshold level of 2. Although the test for independence between loci as implemented in Joinmap is robust against

segregation distortions, it is reassuring that all but one locus (PDfl15a) showed segregation ratios in agreement with the expectations (Table 2-4).

Most loci were amplified in all materials tested with a few exceptions (Fig. 2-1). *Paspalum urvillei* could not be scored for Pdfl7 and Pdfl10. Pdfl7 was not amplified at all in most *P. urvillei* samples, whereas for Pdfl10, weak peaks were observed but could not be reliably scored probably due to interference during PCR from the M13-primed fragment mentioned above. *Paspalum dilatatum* Virasoro showed null alleles for Pdfl18 and Pdfl12a, and *P. dasypleurum* did not produce fragments for Pdfl15. Locus Pdfl22b amplified consistently only in *P. dilatatum* Virasoro and *P. dilatatum* ssp. *dilatatum*.

Table 2-3. Estimated recombination frequency between pairs of loci amplified by the same primer combination, and LOD score for the test of independent segregation between them. Significant threshold LOD=3

Locus pair	Recombination frequency	LOD
Pdfl12a vs Pdfl12b	0.4326	1.09
Pdfl15a vs Pdfl15b	0.3664	0.38
Pdfl20a vs Pdfl20b	0.3665	0.21
Pdfl22a vs Pdfl22b	0.4371	0.03

Table 2-4. Test of the segregation ratios per microsatellite locus. Genotypes coded as: 'a' homozygous *flavescens* allele, 'b' homozygous Virasoro allele, 'h' heterozygous, 'c' dominant allele from Virasoro, and 'd' dominant from ssp. *flavescens*

.Locus	a	h	b	c	d	ratio	chi-square	df	p
Pdfl4	13	22	8	0	0	1:2:1	1.2	2	0.549
Pdfl6	14	18	11	0	0	1:2:1	1.6	2	0.449
Pdfl7	16	17	10	0	0	1:2:1	3.6	2	0.165
Pdfl8	9	22	12	0	0	1:2:1	0.4	2	0.819
Pdfl10	17	20	6	0	0	1:2:1	5.8	2	0.055
Pdfl11	6	21	16	0	0	1:2:1	4.7	2	0.095
Pdfl12a	0	0	15	0	28	3:1	2.2	1	0.138
Pdfl12b	7	28	8	0	0	1:2:1	4.0	2	0.135
Pdfl15a	5	21	17	0	0	1:2:1	6.7	2	0.035
Pdfl15b	10	20	13	0	0	1:2:1	0.6	2	0.741
Pdfl18	0	0	10	0	33	3:1	0.1	1	0.752
Pdfl20a	8	25	10	0	0	1:2:1	1.3	2	0.522
Pdfl20b	14	20	9	0	0	1:2:1	1.4	2	0.497
Pdfl22a	7	25	11	0	0	1:2:1	1.9	2	0.387
Pdfl22b	8	0	0	35	0	3:1	0.9	1	0.343
Pcfl28	12	19	12	0	0	1:2:1	0.6	2	0.741

Variability

All biotypes showed polymorphisms for at least two loci. On the other hand, several alleles were mostly fixed within biotypes but variable among biotypes. Notably Pdfl7 provided excellent biotype-specific markers for this sample (Fig. 2-1c). Pdfl6b was the only invariable locus scored in this sample.

At least two different alleles were cloned and partially sequenced from the focal biotype for Pdfl4, Pdfl6a, Pdfl11, Pdfl12a, Pdfl22a, Pdfl15a, and Pdfl28a. Despite the effect of the long repetitive tract on the quality of the sequence, it can be clearly seen that variation in the focal loci is due to expansion/contraction of the $(GT/CA)_n$ repeat (data not shown).

Despite being the source biotype, *P. dilatatum* ssp. *flavescens* does not display the greatest variability nor allele sizes in this sample (Fig 2-1). The average number of alleles for *P. dilatatum* ssp. *flavescens* is 2.5 with an average size range per locus of 9.9 bp. *Paspalum urvillei* showed a higher number of alleles per locus (3.9) and a higher size range (28.9 bp); furthermore, *P. urvillei* showed polymorphisms for all the scored loci, and all the individuals except 3, 7, and 8 were heterozygous for at least one locus whereas in *P. dilatatum* ssp. *flavescens* only individual 7 appeared heterozygous for locus Pdfl6a. The two individuals sampled from Virasoro and Vacaria were completely homozygous, whereas heterozygosity was observed in both individuals of *P. dasypleurum* (Fig. 2-1e, j, h). The two individuals of *P. dilatatum* ssp. *dilatatum* were heterozygous for all loci except individual 2 at locus Pdfl22b (Fig. 2-1k).

In summary, 19 variable nuclear microsatellite loci were investigated, with one locus monomorphic in all the accessions (Pdfl6b). Among the 18 variable loci, 11 were successfully amplified and interpreted in all the biotypes in this sample. Of the original

12 focal loci, 4 could not be scored in one of the biotypes: Pdfl7 and Pdfl10 in *P. urvillei*, Pdfl18 in Virasoro, and Pdfl15a in *P. dasypleurum*. Three of the non-focal variable loci (Pdfl12b, Pdfl15b, and Pdfl20b) were successfully amplified and scored for all individuals, while Pdfl22b was only amplified in Virasoro, the pentaploids, and one individual of *P. urvillei,* and finally, two loci (Pdfl20-3 and Pdfl28b) were only amplified in the pentaploids.

Chloroplast Variability

All regions were successfully amplified and sequenced except for the *trnT*(UGU)-*trnL*(UAA) spacer for which low-quality sequences were obtained due to the presence of poly-A tracts near both ends (GenBank accession nos. DQ104273-DQ104323). No further efforts were made to improve the quality of the sequences of the (UGU)-*trnL*(UAA) spacer because they were considered appropriate for the purposes of this study. Overall, no sequence variability was found among the biotypes of the Dilatata group except for the length of the poly-A tract in the *trnT*(UGU)-*trnL*(UAA) spacer, and a G-T transversion in the *trnL*(UAA) intron in the Vacaria individual. No repetitive sequences were found except for poly-A/T tracts. Fragment sizes were not variable for any of the loci reported by Provan et al. (2004) or the poly-A repeat located near the *trnL*(UAA)5' exon in the *trnT*(UGU)-*trnL*(UAA) spacer. Only the poly-A repeat located near *trnT*(UGU) (cpDilB) was variable as observed in the original sequences. Fragment lengths (after subtracting the M13 tail) were 198 bp for *P. dilatatum* ssp. *flavescens*, ssp. *dilatatum*, Virasoro, and Vacaria, 199 bp for *P. urvillei* and 197 bp for *P. dasypleurum*. No intrabiotypic variability was observed except for individual 2 in *P. urvillei*, which contained the 198-bp allele.

Discussion

Capture Efficiency

Among the loci reported here, Pdfl2, Pdfl12a, Pdfl15b, and Pdfl20a were found to be compound $(CA/GT)_n$ $(AT/TA)_n$ repeats (Table 2-2). In a genome-wide survey in rice, Temnykh et al. (2000, 2001) found that $(CA)_n$ repeats were frequently associated with $(TA)_n$ repeats.

In genome-wide surveys of grasses, $(CA)_n$ repeats have been reported to be relatively short compared to other dinucleotides (Temnykh et al. 2000). During the enrichment phase of the capture protocol, this may have led to the retention of a limited number of longer repeats, which may explain the high level of redundancy (4/24) of the captured clones. A strong bias towards long repeats may be advantageous because the length of the perfect repeats is expected to be associated with higher degrees of variability (Symonds et al. 2003). Here, loci with more than 30 perfect repeats (Pdfl6, Pdfl15a, Pdfl22, and Pdfl28) revealed the highest number of alleles per locus in both *P. dilatatum* ssp. *flavescens* and *P. urvillei.*

Amplification Profile

Low temperatures during the PCR extension step have been suggested to reduce the generation of frameshift products (commonly known as "stutter"), particularly for fragments containing $(CA)_n$ repeats (Hite et al. 1996). Under standard PCR conditions like those used here, though, extension temperature cannot be reduced below the desired annealing temperature. In preliminary amplifications a noticeable reduction in the number of stutter peaks was observed, particularly for long alleles, when extension was carried out at 53ºC rather than at 72ºC, while even lower temperatures resulted in the production of locus-nonspecific products. However, the use of a single, robust

amplification profile does not require the adjustment of annealing and extension temperatures for each primer pair individually, and greatly increases logistic efficiency when simultaneously working with multiple primer pairs.

Non-Focal Loci

Most studies focus on primer pairs that amplify highly variable single loci. In this study, all bands were taken into account because stable, biotype-specific, co-dominant markers could be extremely useful for hybrid analyses within the complex. Sourdille et al. (2001) analyzed a set of wheat microsatellite primer pairs including primers that amplified more than one locus taking into account their known chromosomal locations and transferability. In that study, 54% of the primer pairs amplified more than one locus, including cases in which the extra bands were monomorphic, independently segregating variable loci or co-segregating linked markers. These results are very similar to the ones obtained in this study, in which 50% of the primer pairs amplified more than one putative locus. In this study, however, extra bands were either monomorphic or independently segregating, but no putative tandem duplications were found. Definitive assessment of homeology would require a genetic map showing that the loci are located in syntenic homeologous chromosome segments.

The number of products amplified was always equal to or less than the number of loci expected for a tetraploid amphiploid, always consistent with the interpretation that primer pairs were amplifying products from either one or the two genomes. Remarkably, primer pair Pdfl20 amplified a number of bands exactly corresponding to the ploidy level in heterozygous individuals.

Nuclear Loci

At least two variable loci were identified for each of the biotypes including the three sexual tetraploids represented by only two individuals. It is likely then that if more individuals were analyzed, this set of loci could contain useful markers for population structure and breeding system assessment of all the biotypes in the group. Overall, the focal species showed less variation than *P. urvillei*. Only in 25% of the loci (Pdfl4, Pdfl6a, Pdfl20a, and Pdfl28) did *P. dilatatum* ssp. *flavescens* show consistently longer repeats than *P. urvillei*. It is typically expected that due to selection for long repeats during library enrichment, longer repeats and higher variability are more likely to be found in the focal biotype (Ellegren et al. 1995), an artifact known as ascertainment bias. However, *P. urvillei* consistently showed more variability and a much higher level of heterozygosity for most loci. A higher number of alleles was captured in *P. urvillei*, even at loci for which the average fragment length was clearly lower than in *P. dilatatum* ssp. *flavescens* (Fig 2-2 g, j) which contradicts the accepted consensus that repeat number and variability are associated regardless of the causes of this correlation (Schlöterer 2000). A statistical comparison of variability within different biotypes is beyond of the scope of this paper; however, the clear differences in variability and heterozygosity between *P. dilatatum* ssp. *flavescens* and *P. urvillei* are likely to be real despite the small sample size presented here. Amos et al. (1996) claim that heterozygosity may lead to an increase in mutation rates at microsatellite loci. This may seem to be the case when comparing *P. dilatatum* ssp. *flavescens* and *P. urvillei*; however, the stochastic effects of the restricted distribution of *P. dilatatum* ssp. *flavescens* and its apparently extreme selfing rate may deserve further investigation as putative explanations for the observed “reverse ascertainment bias”. The relative distance between the species analyzed should also be

taken into account to interpret meaningfully cross-amplification and ascertainment bias. The I, J, and X genomes within the Dilatata polyploids can be considered to represent different species because they are implicitly assumed to have diverged independently as different diploid species between their coalescence time and the polyploidization event that brought them back together. When the putative homeologous sequences shown in Fig.2-2 are compared, a strong ascertainment bias is evident in all of them. Similar flanking sequences combined with shorter and imperfect repeats like those found in the non-focal loci in this study were found by Chen et al. (2002) when they amplified microsatellites developed for *Oryza sativa* in congeners containing different genomes.

Variation was also found between the two individuals of *P. dilatatum* ssp. *dilatatum*. These two individuals share 13 heterozygous allele combinations, making it very unlikely that the three differences found (Pdfl15a and Pdfl22b, Fig. 2-1 h and k, respectively) are due to a sexual recombination event or independent origins. However, enough mutations seem to have accumulated in this clonal biotype to observe variability with this set of microsatellite loci.

Alleles found in *P. dilatatum* ssp. *dilatatum* in eight loci are also present in Virasoro, suggesting that this tetraploid could have been involved in the origin of the pentaploid biotype. The pentaploids could not have arisen directly from a cross involving the Virasoro genotypes analyzed here because the pentaploids are heterozygous for loci for which Virasoro shows null alleles (Pdfl12a and Pdfl18). More intra-biotypic variability must be analyzed, but the markers developed in this study seem to have great potential for assessing the relationships among the sexual and apomictic components of the Dilatata group.

Clustering and uneven genomic distribution of $(CA)_n$ motifs has been reported in several genomes (Elsik and Williams 2001, Schmidt and Heslop-Harrison 1996); however, no close linkage was detected among the loci analyzed in this study. Any subset of these loci can then be chosen for a specific application based on amplification consistency and variability to provide independent characters.

Chloroplast Microsatellites

Even though cpDilB was the only variable chloroplast microsatellite identified, it could potentially be very informative for assessing hybridization among biotypes because the chloroplast genome is inherited as a single cohesive group and different alleles are fixed within biotypes. *Paspalum urvillei* is the most widespread of the sexual members of the Dilatata group, and its current range overlaps with those of the rest of its members. Putative hybrids can be confirmed or *P. urvillei* can be ruled out from being the female progenitor by using this marker. In this sample, individual 2 of *P. urvillei* was the only one that showed a chloroplast allele that is not typical of its biotype. This accession was indeed collected near the area of co-occurrence with biotype Vacaria with which *P. urvillei* has been reported to hybridize (Valls and Pozzobon 1987). Our ability to identify this putative hybrid confirms the utility of this marker.

Conclusions

Nuclear and chloroplast markers are reported with potential applications in population genetics and phylogenetic studies within the Dilatata group. Highly variable nuclear markers can be used to address population structure and breeding system issues for all the biotypes in the group. On the other hand, more stable biotype-specific loci may be used as co-dominant markers to assess the relationships among biotypes and particularly the origin of the apomictic components of the complex. The variable

chloroplast microsatellite locus reported may in turn provide valuable information about the relationship between the most widespread sexual member of the complex (*P. urvillei*) and the rest of the biotypes.

CHAPTER 3
BREEDING SYSTEM AND POPULATION GENETIC STRUCTURE OF *Paspalum dilatatum* ssp. *flavescens* (POACEAE).

Introduction

Paspalum dilatatum Poir. is a warm-season grass native to the grasslands of temperate and subtropical South America. The species includes several tetraploid, pentaploid and hexaploid forms. The common pentaploid form (*P. dilatatum* ssp. *dilatatum*) was one of the first warm-season grasses to be cultivated for pasture (Skerman and Riveros 1992), however, its commercial use has been limited by poor seed production and susceptibility to ergot (*Claviceps paspali*). The pentaploid biotype reproduces by apomixis (Bashaw and Holt 1958) and it has been assigned the IIJJX genomic formula (Burson 1983). Because no sexuality has been reported in this subspecies and collections have not provided much variability for the characters of interest, efforts were soon undertaken to elucidate its relationships with other *Paspalum* species in the hope that a breeding strategy could be devised. As a consequence of extensive collections and cytogenetic investigations in *P. dilatatum* and related species, three sexual tetraploid biotypes with the IIJJ genomic formula have been identified and are usually included in the species (*P. dilatatum* ssp. *flavescens* Roseng. Arr. et Izag., and biotypes Virasoro and Vacaria). Burson (1983) proposed that recombinant IIJJX pentaploids could be synthesized by crossing one of the sexual biotypes and an apomictic hexaploid with the IIJJXX genomic formula. This genomic formula was later assigned to the hexaploid biotypes Chirú and Uruguaiana (Burson 1991, 1995), and synthetic

pentaploids have been readily produced (Burson 1991b, 1995, Speranza 1994 unpubl. res.) and show excellent forage potential. As only one population is known for Chirú, and probably both Chirú and Uruguaiana are very likely a single clone, genetic variability for the breeding scheme mentioned above must come from the tetraploid parent. Among the tetraploids, *P. dilatatum* ssp. *flavescens* is morphologically the most similar to the common pentaploid biotype. The forage potential of *P. dilatatum* ssp. *flavescens* itself has been assessed and compared with that of other biotypes. Besides a lower production potential, this biotype's production is more concentrated in the spring (Millot 1969). *Paspalum dilatatum* ssp. *flavescens* produces more seed than the pentaploids and it is thought to be more resistant to *Claviceps*. *Paspalum dilatatum* ssp. *flavescens* is currently distributed in southern Uruguay and the eastern province of Buenos Aires, Argentina (Rosengurtt et al. 1970). *Paspalum dilatatum* ssp. *dilatatum* is also found throughout the range of *P. dilatatum* ssp. *flavescens*. The tetraploid subspecies can be distinguished from the pentaploids by its more erect and usually taller culms, larger, more rounded spikelets, and yellow rather than purple anthers (Fig. 3-1). Recently, microsatellite data for a small sample of this biotype suggested a high level of homozygosity (Chapter 2)

A collection of *P. dilatatum* ssp. *flavescens* was deposited at the Germplasm Bank of the Facultad de Agronomía in Montevideo, Uruguay during the 1990s. An efficient use of this collection will depend on knowledge about its genetic structure, the breeding system of the species, and its geographical structure (Epperson 1990, Rao and Hodgkin 2002). No morphological qualitative markers have been identified in the biotype, and there is no evaluation of the degree of genetic variability it contains or its structure. In

this study, a sample of this collection is analyzed using six microsatellite loci, and the breeding system, genetic structure, and geographical structure of the genetic variability are discussed.

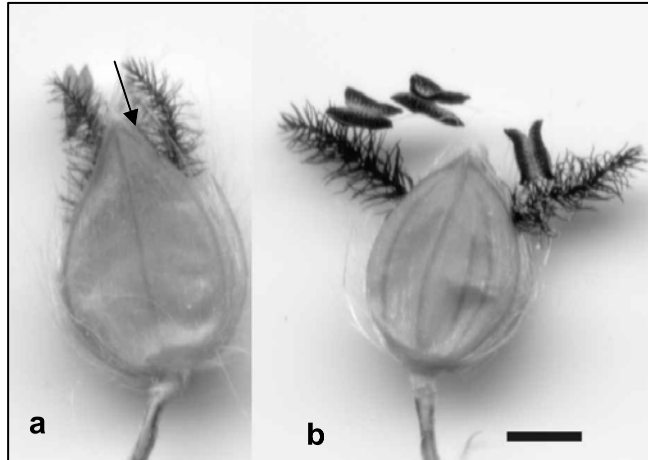

Fig. 3-1. A spikelet of *P. dilatatum* ssp. *flavescens* (a) and *P. dilatatum* ssp. *dilatatum* (b). Note the relatively shorter, less hairy stigmata in *P. dilatatum* ssp. *flavescens* and trapped anthers (arrow). The bar represents 1 mm.

Materials and Methods.

Collection Strategy

Because tetraploid and pentaploid plants are practically indistinguishable in grazed pastures, most collections were made on major roads in southern Uruguay. Based on field and greenhouse observations that suggested autogamy, an emphasis was made on collecting seed from a single panicle from each individual while sampling as many individuals as possible. *Paspalum dilatatum* ssp. *flavescens* grows in dense patches on roadsides and in hilly areas, and patches are typically restricted to lower slopes excluding the bottom. Each population consisted of up to four patches including both sides of the road and both sides of a waterway when one was present. Seeds were deposited in the Germplasm Bank at the Facultad de Agronomía in Montevideo, Uruguay. For most

accessions, a voucher specimen was also deposited at the herbarium of the Facultad de Agronomía (MVFA).

Seed samples of 21 accessions were later retrieved from the Germplasm Bank for this study. Accession numbers are given in Table 3-1, and collection localities can be seen in Figs. 3-3 and 4. Eight individuals per accession were grown in greenhouse conditions. All individuals were observed during the reproductive stage to ensure that no pentaploids were present.

Microsatellite Amplification

DNA was extracted from fresh leaves using Sigma Genelute^{TM} kit (Sigma-Aldrich, St. Louis, MO). PCR and separation were carried out as described in (Chapter 2) using extended forward primers with an M13 tail (Boutin-Garnache et al. 2001). Briefly, 0.5-3 μL of DNA were added to a PCR mix consisting of 2 units of NEB Taq polymerase (New England Biolabs, Beverly, MA), 1.5 mM $MgCl_2$, 0.15 μM of the reverse primer and labeled M13 primer, 0.01 μM of the extended forward primer, and 0.1 mM of each dNTP in the manufacturer's buffer. Amplification was carried out with the same two-step program for all primer pairs consisting of a denaturation step of 15 s at 94ºC and an annealing/extension step of 3 m at 53ºC (Chapter 2). Primer pairs Pdfl4, Pdfl6, Pdfl12, Pdfl15 and Pdfl20 (Chapter 2) were selected for this study. Three different fluorochrome labels were used (D2, D3 and D4, Beckman Coulter, Fullerton, CA) for the M13 primers. PCR products labeled with D2, D3 and D4 were combined 5:3:2, and 1 μL of the combined products was loaded on a CEQ 8000 capillary sequencer (Beckman-Coulter, Fullerton, CA). Chromatograms were visualized on CEQ^{TM} Genetic Analysis system software (Beckman Coulter, Fullerton, CA) and scored manually.

Data Analysis

AMOVA (Excoffier et al. 1992) was implemented with Arlequin (Schneider, Roessli, and Excoffier 2000) using both the number of different alleles (F_{ST}) and the sum of squared size difference (R_{ST}) as a distance measure (Slatkin 1995). Average squared distances (D_1) (Goldstein et al. 1995, Slatkin 1995) and the proportion of shared alleles (D_{ps}) (Bowcock 1994) as implemented in MSAanalyzer (Dieringer and Shlöterer 2002) were used to measure population differentiation. D_1 was chosen because it takes into account the mutation history reflected by allele size similarities caused by the stepwise mutation mechanism typical of microsatellite loci. Furthermore, D_1 is expected to reflect linearly the divergence time between populations even when averaged across loci with different mutation rates (Goldstein et al. 1995). The calculation of D_1 makes use of intrapopulational allele size variance; when calculation is based only on average allele size as is commonly implemented for $(\delta\mu)^2$ (Goldstein et al. 1995), the distance between populations including a combination of long and short alleles and populations with only intermediate alleles is severely underestimated, and this situation was often approached in the current data set.

Population parameters of inbreeding (F_{IS}) and gene diversity (H_e) were calculated with Genepop (http://wbiomed.curtin.edu.au/genepop/).

A Mantel test (Mantel 1967) was implemented using Passage (Rosenberg 2001) between matrices of D_1 and D_{ps} genetic distances and geodesic distances between populations to detect isolation by distance (Heywood 1991, Slatkin and Arter 1991). Geodesic distances between populations were generated by Passage from the original population geographical coordinate list. Cluster and principal component analyses were performed with MVSP (KCS, Anglesey, Wales).

Results

All primers amplified the expected products in all individuals. Lack of amplification in a first attempt was overcome by varying the amount of DNA used in the PCR reaction in all cases. Although all DNAs had been extracted with the same protocol, ten-fold variations in the amount of DNA were sometimes necessary to achieve satisfactory amplification. No homozygotes for null alleles were thus detected, and given the levels of homozygosity found, it is not likely that they are present as heterozygous combinations in this sample.

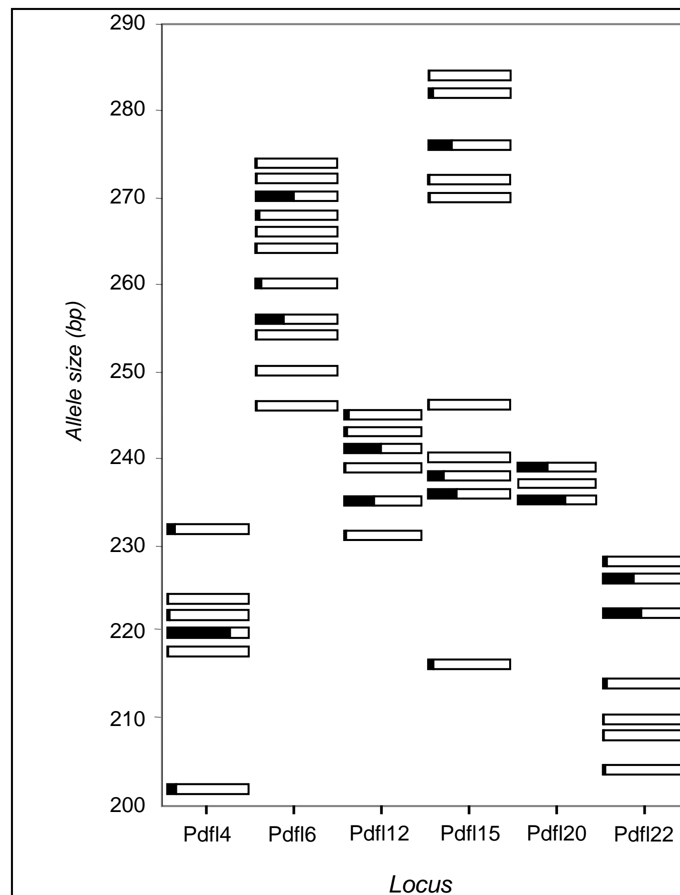

Fig. 3-2. Allele size distribution for six microsatellite loci in *P. dilatatum* ssp. *flavescens*. Shaded areas represent relative allele frequencies.

Table 3-1. Genetic diversity and heterozygosity for individual populations of *P. dilatatum* ssp. *flavescens* for 6 microsatellite loci.

						Pdfl20			Pdfl4			Pdfl12			Pdfl15			Pdfl22			Pdfl6		
Pop.[a]	Acc.[b]	Gen[c]	Het[d]	F_{is}[e]	H_e[f]	A[g]	Het	Rep[h]	A	Het	Rep	A	Het	Rep	A	Het	Rep	A	Het	Rep	A	Het	Rep
101	7494	2	0	1.00	0.04	1	-	39	1	-	30	1	-	43	1	-	65	1	-	46	2	0	(42-45)
102	7495	7	3	0.67	0.57	2	2	(37-39)	4	2	(21-36)	2	2	(40-43)	2	1	(43-44)	4	1	(34-43)	5	1	(45-54)
50	7363	4	1	0.92	0.30	2	1	(37-39)	1	-	30	2	0	(43-44)	2	0	(42-43)	2	0	(43-45)	2	0	(50-52)
57	7364	4	0	1.00	0.47	2	0	(37-39)	2	0	(21-36)	2	0	(40-43)	2	0	(43-47)	2	0	(43-45)	2	0	(45-52)
62	7429	7	0	1.00	0.52	2	0	(37-39)	2	0	(21-30)	2	0	(40-43)	3	0	(42-62)	3	0	(37-45)	2	0	(45-52)
64	7432	6	1	0.93	0.30	2	0	(37-39)	2	0	(30-36)	1	-	43	4	0	(42-62)	2	1	(39-43)	1	-	52
65	7435	7	2	0.87	0.32	2	0	(37-39)	1	-	30	2	1	(43-44)	3	1	(59-66)	1	-	43	3	0	(51-54)
66	7433	4	1	0.78	0.19	2	0	(37-39)	2	0	(30-36)	2	1	(40-42)	1	-	32	1	-	43	2	1	(45-52)
68	7434	5	1	0.96	0.53	2	0	(37-39)	2	1	(21-30)	2	0	(40-43)	3	0	(42-62)	2	0	(43-45)	3	0	(45-52)
7	7355	6	3	0.79	0.31	1	-	37	2	2	(30-36)	2	1	(40-43)	2	0	(43-62)	1	-	43	2	0	(51-52)
72	7438	7	0	1.00	0.40	1	-	39	2	0	(30-36)	3	0	(40-43)	2	0	(43-62)	2	0	(39-43)	3	0	(40-52)
76	7467	5	0	1.00	0.30	2	0	(37-39)	2	0	(30-31)	1	-	40	2	0	(42-62)	2	0	(43-45)	3	0	(45-52)
78	7469	4	0	1.00	0.19	2	0	(37-38)	1	-	30	2	0	(40-42)	1	-	42	1	-	45	2	0	(44-45)
79	7470	3	0	1.00	0.35	2	0	(37-39)	2	0	(30-36)	2	0	(40-43)	3	0	(32-62)	2	0	(39-43)	1	-	52
82	7473	3	0	1.00	0.23	2	0	(37-39)	3	0	(21-36)	2	0	(38-43)	2	0	(32-62)	1	-	43	1	-	52
87	7478	4	0	1.00	0.33	2	0	(37-39)	3	0	(21-36)	2	0	(38-43)	2	0	(32-62)	1	-	43	1	-	52
91	7481	7	3	0.73	0.39	2	1	(37-39)	1	-	30	3	2	(40-44)	2	0	(42-62)	2	1	(43-45)	3	1	(45-53)
92	7482	2	1	0.80	0.10	1	-	37	1	-	30	1	-	40	2	0	(42-62)	2	0	(43-45)	2	1	(45-47)
95	7485	3	0	1.00	0.21	1	-	37	1	-	30	2	0	(43-45)	2	0	(62-65)	2	0	(34-43)	2	0	(45-47)
98	7491	4	0	1.00	0.16	2	0	(37-39)	2	0	(21-32)	1	-	45	1	-	42	1	-	45	2	0	(52-53)
99	7492	1	-	-	0.00	1	-	37	1	-	30	1	-	40	1	-	42	1	-	45	1	-	45
						Ave.	H_e		Ave	H_e			H_e		Ave	H_e		Ave	H_e		Ave	H_e	
Totals		80	16	0.92	0.59	1.7	0.49	(37-39)	1.8	0.39	(21-36)	1.8	0.62	(38-45)	2.0	0.75	(32-66)	1.7	0.62	(34-46)	2.1	0.66	(40-54)

[a] Population label. [b] Accession number. [c] Number of genotypes per population. [d] Number of heterozygous individuals. [e] multilocus fixation index. [f] Expected heterozygosity. [g] Number of alleles. [h] number of repeat units in the microsatellite inferred from allele length.

All loci were variable, providing high expected heterozygosities (Table 3-1). Pdfl4 was the least variable locus (H_e= 0.39) and the only one to show a clearly unimodal allele size distribution with one allele showing a frequency of 0.77 (Fig. 3-2).

Allele size ranges and their frequencies for the whole sample are shown in Fig. 3-1. Allele size distributions are clearly bimodal for Pdfl6, Pdfl12, Pdfl15, and Pdfl20. Consequently, in spite of the high proportion of alleles with very low frequencies, H_e values for all loci across populations are rather high (Table 3-1). Besides showing bimodal distributions, not all possible allele sizes are represented. Three considerable gaps were found: a 20-bp gap in Pdfl4 and two gaps of 20 and 24 bp, respectively, in Pdfl15.

The sample contained a high level of overall homozygosity; only 16 individuals (9.5%) showed any heterozygosity. However, only 4 populations contained more than a single heterozygote, and notably only 4 individuals were heterozygous at more than one locus, three of them from population 102. In turn, those three individuals share all the segregating alleles, making it possible that they may belong to a single segregating progeny (data not shown). Eleven populations (52%) contained no heterozygotes, and fixation indices within individuals (F_{IS}) for the rest of the populations are high, including those for population 102 (0.673). The average F_{IS} value is 0.916. In spite of the low number of heterozygotes, intrapopulational variability is considerable, with about half of the populations reaching H_e values above 0.35. Although one population (99) contained a single genotype, the average number of genotypes is 3.8 per populational sample of only 8 individuals. The average number of alleles per locus within populations is 1.8,

ranging between 1.7 and 2.1 (Table 3-1). Then, despite differences in variability, all loci contributed allele size differences which would detect outcrossing if it were taking place.

Allele size distribution between populations varied among loci; however, for some loci, particularly Pdfl6 and Pdfl15, several variable populations showed non-overlapping ranges (Table 3-1). This observation suggests that there may be structure in the allele size range within populations, which could shown by using a distance method that takes allele size into account.

Table 3-2. AMOVA of a six-microsatellite-locus data matrix for 21 populations of *P. dilatatum* ssp. *flavescens*.

Distance measure R_{ST} Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	20	28961.05	86.62 Va	58.27
Within populations	315	19537.88	62.03 Vb	41.73
Total	335	48498.93	148.65	
Fixation Index	F_{ST} :	0.583		
Significance test (1023 permutations)	P(rand. value >= obs. value) = 0.00000+-0.00000			

Distance measure F_{ST} Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	20	330.14	0.98Va	54.04
Within populations	315	262.44	0.83 Vb	45.96
Total	335	592.58	1.813	
Fixation Index	F_{ST} :	0.54		
Significance test (1023 permutations)	P(rand. value >= obs. value) = 0.00000+-0.00000			
Significance test (1023 permutations)	P(rand. value >= obs. value) = 0.00000+-0.00000			
Va and F_{ST} :	P(rand. value >= obs. value) = 0.00000+-0.00000			

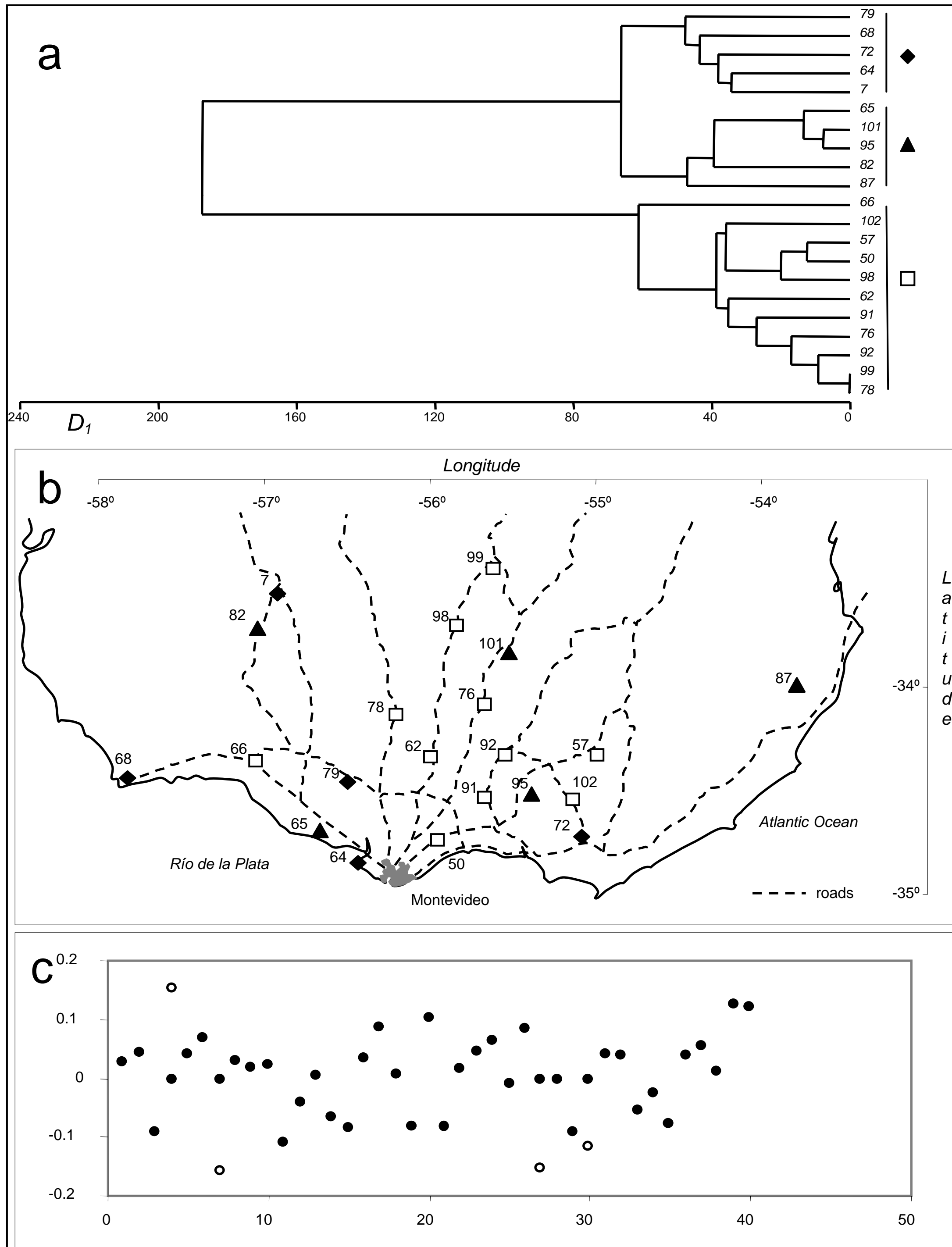

Fig. 3-3. Genetic distances among 21 populations of *P. dilatatum* ssp. *flavescens* and their geographical distribution. a. Complete linkage phenogram of D_1 distances. b. Geographical distribution of the clusters shown in a (see symbols). Only roads on which the samples were collected are shown. c. a Mantel correlogram of D_1 genetic distances and geographical distance between populations. Open circles show significant correlations.

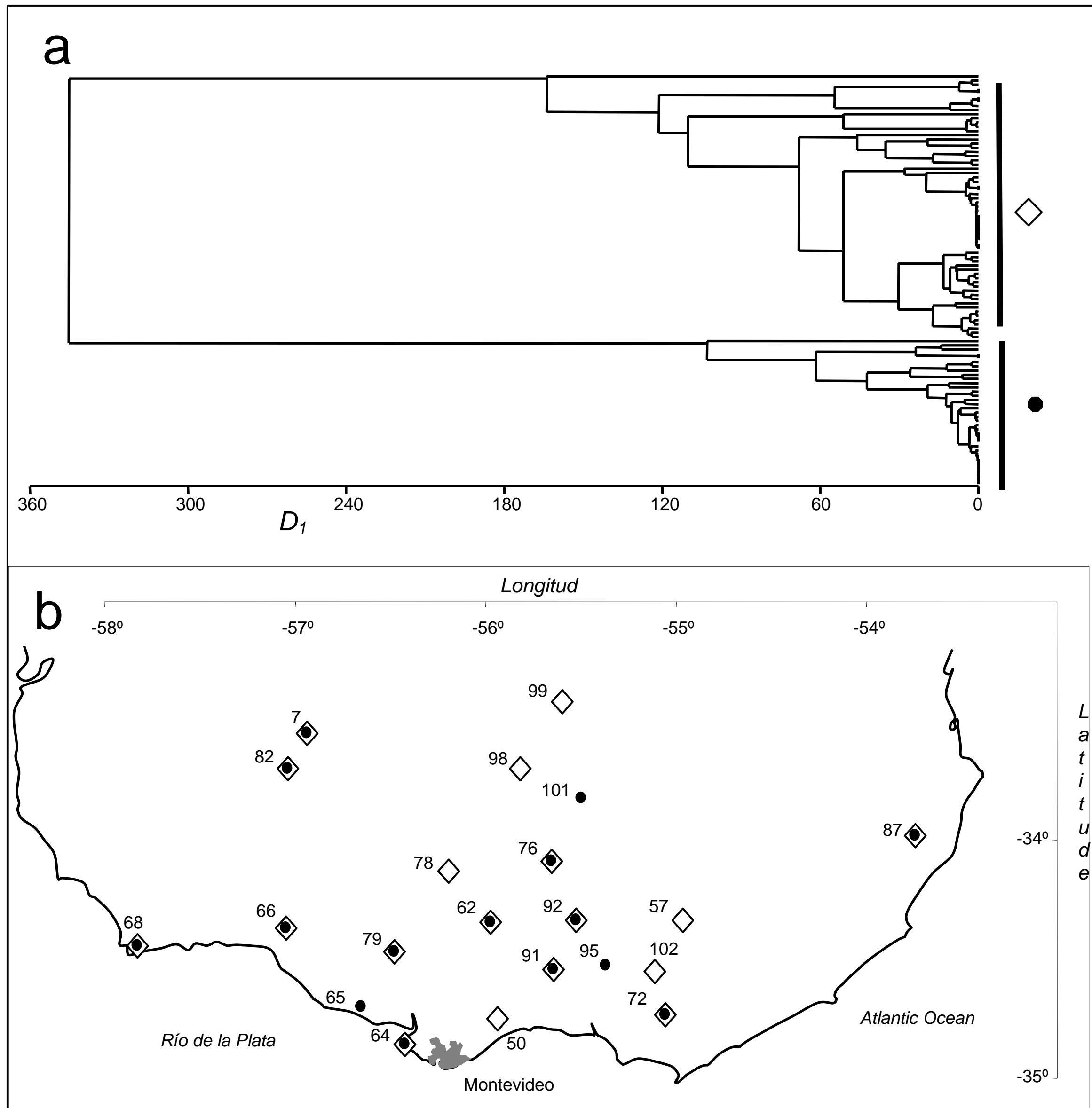

Fig. 3-4. Genetic distances among individual genotypes of *P. dilatatum* ssp. *flavescens* and their geographical distribution. a. Average linkage (UPGMA) phenogram of D_1 distances. b. Geographical distribution of the clusters shown in a (see symbols in a).

Population subdivision as analyzed by AMOVA produced F_{ST} values of 0.58275 when using the average allele size difference as a distance measure, and 0.54041 when using the number of shared alleles. Both estimates were highly significant (Table 3-2).

D_1 genetic distances between populations were calculated and the distance matrices used for cluster analysis. Several linkage methods were used and all resulted in similar

clusters (results not shown). One of the best resolved phenograms is shown in Fig. 3-3a. The resulting cluster memberships were plotted on a map (Fig. 3-3b) to visualize any possible geographical structure. Mantel tests were also performed comparing the D_1 and D_{ps} distance matrices to the geographical distance matrix. None of these tests was significant. Mantel correlograms were also produced which showed no association between geographical and genetic distance with either distance measure. One of those correlograms is shown in Fig. 3-3c.

A similar approach was followed to analyze possible geographical structure in the distribution of individual genotypes. A UPGMA phenogram of D_1 genetic distance between pairs of individual genotypes is shown in Fig. 3-4a, and the geographical distribution of the two main clusters is shown in Fig. 3-4b. D_{ps} produced similar results (data not shown). The two main clusters plotted in Fig. 3-4b not only show a complete lack of geographical segregation, but they also show extensive overlap.

Discussion

Mating System

The observed inbreeding rate as reflected by the fixation index (F_{IS}) depends not only on autogamy but also on pollinations between flowers from the same inflorescence (geitonogamy), flowers on different ramets of the same genet (tillers), and, finally, the genetic substructuring of the populations which increases biparental inbreeding (Ritland 1984, Brown 1989). While all these effects are confounded in populational data, as in our case, there are indications that the selfing rate itself is very high.

The high intrapopulational genotypic differentiation observed in *P. dilatatum* ssp. *flavescens*, coupled with extremely high fixation indices (F_{IS}≈0.97), suggests a lower contribution of biparental inbreeding to the observed homozygosity in relation to the

actual selfing rate. Extensive observation of florets of this species during manual hybridization suggests two mechanisms for such a high selfing rate. First, *P. dilatatum* ssp. *flavescens* shows several characteristics that are typical of cleistogamous grasses (Cambpell et al. 1983): stigmata and stamen filaments are relatively short in reference to floret dimensions when compared to other biotypes of the species, and anther dehiscence occurs immediately after or during anthesis. Furthermore, one, two or all three anthers have been often observed to remain within the floret during and after anthesis, while stigmata barely protrude outside the spikelet. Frankel and Galun (1977) classified this mechanism as functional autogamy, and it has frequently been observed in grasses. The second mechanism involves anthesis prior to emergence of the panicle from the sheath; this has been more frequently observed for the first one or two racemes of the panicle. Campbell et al. (1983) listed this mechanism as *Type Ia* and considers that cleistogamy in these cases may be mostly an environmental response. In agreement with field and greenhouse observations in *P. dilatatum*, factors such as light, temperature and moisture have been reported to affect anthesis and panicle emergence (Campbell et al. 1983). For a warm-season grass with an extended reproductive phase like *P. dilatatum*, environmental responses could cause wide seasonal variations in selfing rates. Delayed anthesis and faster culm elongation in the early spring under cool temperatures and no moisture restrictions may increase the probability of outcrossing.

Genetic Differentiation and Geographical Structure

Genetic differentiation overall is very high in *P. dilatatum* ssp. *flavescens*. F_{ST} estimates for continuous populations in which autogamy is thought to be the main factor contributing to structuring have been reported to vary between 0.29 and 0.78 (Heywood 1981). The current sample of *P. dilatatum* ssp. *flavescens* was mostly collected in

disturbed environments such as roadsides, and seed morphology suggests dispersion by gravity. These two factors, together with the observed selfing rates, contribute to a high expectation for population subdivision (Loveless and Hamrick 1984). Hamrick and Godt (1990) estimated a proportion of total genetic diversity among populations of 0.51 for 78 selfing species analyzed with isozymes, whereas their estimates for outcrossing species vary between 0.1 and 0.2 depending on the mode of seed dispersion. Our F_{ST} estimate of 0.54 is therefore high and reflects the biological characteristics of the species. In our data set, non-overlapping allele size distributions in variable populations suggest the presence of informative non-random distribution of allele sizes. Regardless of the exact evolutionary model each microsatellite locus follows, the process seems to retain some mutational memory that can be captured by R_{ST} much more efficiently than F_{ST} with the resulting underestimation of overall genetic differentiation by the latter (Slatkin 1995). In this case, however, the use of R_{ST} instead than F_{ST} as a distance measure in the AMOVA did not significantly increase the estimate as expected. There are several possible reasons for this. F_{ST} and R_{ST} estimates of genetic subdivision would converge in situations where mutation plays a minor role in population differentiation in relation to drift (Slatkin 1995). Population and mating system dynamics leading to high levels of genetic drift and recent long-distance dispersal of individuals or population expansion will then improve the performance of F_{ST} relative to R_{ST} (Slatkin 1995, Estoup et al. 1998).

Under a strict stepwise mutation model, gaps in the allele-length distributions are expected after severe drift, like that caused by small local effective population sizes or founder effects followed by a rapid expansion of the population. With time, mutation is expected to fill the gaps between the sampled alleles (Cornuet and Luikart 1996). Not

only were some large gaps detected in this sample, but also, in ranges in which allele sizes are rather continuous, the distributions were multimodal, with very low frequencies for the intervening length categories. It could be argued that microsatellites have not been observed to follow a strict stepwise mutation model. It seems reasonable to assume that a mixed model (Di Rienzo 1994) is most realistic. Direct observation of microsatellite mutations in maize has shown a majority of single-step length increase events and fewer greater downward mutations (Vigouroux 2002). Observed allele-size frequency distributions in self-pollinated *Arabidopsis thaliana* show continuous, mostly unimodal distributions, especially for loci with a high number of perfect repeats (Symonds and Lloyd 2003). In *P. dilatatum* ssp. *flavescens*, loci with very long perfect repeat tracts like Pdfl15 and Pdfl6 (Chapter 2) show clearly discontinuous multimodal distributions. It follows then that observed mutation patterns alone would not fully explain the allele-size distributions observed in this sample.

The lack of correlation between geographical and genetic distance also supports a scenario in which drift or long-range dispersal dominates the genetic dynamics of the population. No correlation or clear geographical pattern was observed with population or individual genotype data. Furthermore, the geographical distribution of identical genotypes shared by several populations did not show geographical clustering (data not shown).

It has been shown, particularly in selfing grasses, that molecular marker diversity, either allozymes or microsatellites, could reflect the effects of selection (Allard 1972, Nevo 1998, Li 2000). Rare hybridization events or long-distance dispersal can trigger rapid genotype turnover at a particular site or microsite. Seemingly continuous

populations may then harbor widely divergent locally adapted genotypes whose relatedness would not be reflected by neutral molecular markers. Green et al. (2001), working at a more detailed spatial scale than in this study, found admixtures of highly divergent microsatellite genotypes in *Anisantha sterilis*, a selfing weedy grass. Selection, migration and temporal variation in the mating system were invoked as possible causes for the lack of spatial genetic structure and persistence of divergent genotypes within populations.

The great majority of our accessions were collected on roadsides; for two main reasons: first, no dense stands of this subspecies are commonly found in other environments, and second, *P. dilatatum* ssp. *dilatatum* is found almost continuously in nearly all areas in Uruguay and almost always where *P. dilatatum* ssp. *flavescens* is found. Distinguishing between subspecies in the field is not easy unless panicles are present. Except for vacant lots in urban areas, most of the country is under grazing by cattle, and panicles of *P. dilatatum* ssp. *flavescens* are seldom observed under grazing. In fact, *P. dilatatum* ssp. *flavescens* may be largely excluded by grazing. Road construction, shoulder leveling and periodical mowing of the roadsides are three types of events that can strongly affect population dynamics. Construction may provide an opportunity for long-distance dispersal or severe local bottlenecks. The plant cover of a road construction site is completely cleared, creating open environments for colonization. The seeds of the future colonizers may be brought from variable distances depending on the origin of the gravel that is used or the application of turf patches to the barren slopes that are created. Particularly in secondary roads, many stands of this subspecies extend into the gravel shoulders and even into minor cracks in the asphalt itself. Shoulder leveling in

these areas necessarily involves uprooting and dragging whole or big portions of *P. dilatatum* ssp. *flavescens* stands over variable distances. On main roads, the roadsides also are periodically mowed with inevitable movement of panicles and seeds. From a long-term perspective, species composition in Uruguayan grasslands has been greatly influenced by the introduction of cattle in the 17th century. Rodríguez et al. (2003) have shown that during a ten-year grazing exclusion, dominant grass types in a Uruguayan grassland rapidly shifted from prostrate warm-season species to cool-season erect types with narrow leaves and bigger seeds. Grazing patterns must have been changing continuously due to the gradual replacement of Pampas deer by cattle. Pampas deer (*Ozotoceros bezoarticus*) were still abundant at the beginning of the 19th century (Darwin 1839), while they are currently restricted to only two small populations in the whole country (González et al. 1998). Altesor et al. (1998) have in turn reported dramatic floristic change for the same site during 55 years under continuous grazing. It is clear that the impact of grazing in Uruguay during the last four centuries must have been and still is strong, particularly on erect grasses like *P. dilatatum* ssp. *flavescens*. It can be hypothesized that the subspecies may have suffered a strong bottleneck after the introduction of cattle followed by relatively recent recolonization of the fenced roadsides. This event, coupled with the present short-term effects of roadside habitats on population dynamics and dispersal, sufficiently accounts for the lack of geographical structuring, lack of mutational memory evidenced by the low R_{ST} values, and discontinuous allele size distributions.

CHAPTER 4
EVOLUTIONARY RELATIONSHIPS AND MECHANISMS IN THE DILATATA GROUP (*PASPALUM*, POACEAE)

Introduction

The genus *Paspalum* contains approximately 350 to 400 species (Clayton and Reinvoize 1986) and has traditionally been divided into informal groups (Chase 1929). The Dilatata group of *Paspalum* contains several species with great forage potential, and several of them have been used as forage crops (Skerman and Riveros 1992). *Paspalum dilatatum* Poir. and its related species are warm-season grasses native to the grasslands of temperate South America and they are well adapted to resist frequent frosts during the winter (Burson et al. 1991, da Costa and Scheffer-Basso 2003). This environmental adaptability has allowed some members of the group, particularly *P. dilatatum* ssp. *dilatatum* and *P. urvillei* Steud., to reach worldwide distributions wherever a warm-temperate climate combined with sufficient rainfall exist.

The members of the Dilatata group have been classified into several formal and informal taxonomic categories that will be referred to as biotypes in this study. The common biotype of *P. dilatatum* (*P. dilatatum* ssp. *dilatatum*) is a complex apomictic pentaploid hybrid, and efforts to identify its putative ancestors have led, over several decades, to the accumulation of abundant cytogenetic information about the relationships among all the species and biotypes within the Dilatata group. The conclusions of a whole era of cytogenetic analysis based on meiotic studies in interspecific hybrids are summarized in Table 4-1.

Table 4-1. Genomic formulae and reproductive systems of the members of the Dilatata group

Species or biotype	2n (x=10)	Genomic Formula	Author*	Reproductive system**
P. dilatatum ssp. *dilatatum*	50	IIJJX	Burson (1983)	Apomictic (Bashaw and Holt 1958)
P. dilatatum ssp. *flavescens*	40	IIJJ	Burson et al. (1973)	Sexual
P. dasypleurum	40	IIJJ	Quarín et al. (1995)	Sexual
P. urvillei	40	IIJJ	Burson (1979)	Sexual
P. dilatatum Virasoro	40	IIJJ	Caponio et al. (1990)	Sexual
P. dilatatum Vacaría	40	IIJJ	Quarín et al. (1993 unpub).	Sexual
P. dilatatum "Chirú"	60	IIJJXX	Burson (1991)	Facultative apomict (Burson et al. 1991, Millot 1977)
P. dilatatum Uruguaiana	60	$IIJJXX_2$	Burson (1992)	Apomictic (Burson et al. 1991)
P. dilatatum Torres	60	?		Apomictic (Burson et al. 1991)
P. pauciciliatum	40	AA_1BC	Moraes Fernandes et al. (1968)	Apomictic

* Authorities are given for the publication of the genomic formula.
**Authorities are given for works that specifically addressed the reproductive system. All sexual biotypes have been crossed, and sexuality is well established.

At least two attempts have been made previously to present the Dilatata complex in a comprehensive way, including hypotheses on the relationships among its members. Moraes Fernandes et al. (1968) did not provide genomic formulae but deduced genomic architectures from meiotic behavior. They represented the genomic architectures in terms of the number of copies of entire genomes which were assigned letters that do not necessarily signify homology across biotypes. A number of biotypes were described in this work, including the pentaploid apomictic common form (*P. dilatatum* ssp. *dilatatum*), the Uruguaiana and Torres hexaploid biotypes, *P. pauciciliatum* and the sexual tetraploid biotype of *P. dilatatum* now known as Vacaria. The latter was not differentiated from the “yellow anthered” form (*P. dilatatum* ssp. *flavescens*) which had previously been described by Bashaw and Forbes (1958). The common pentaploid biotype (AABBC) was hypothesized to have arisen as a 2n+n hybrid involving a

tetraploid form and a diploid genome donor, or alternatively, as the product of an allotetraploid and an allohexaploid sharing the A and B genomes. The hexaploid Uruguaiana (AAA_1A_1BB) was proposed to be a 2n+n hybrid between two tetraploids, and *P. pauciciliatum* (AA_1BC) was hypothesized to be a hybrid between two tetraploids with one partially homologous genome. Finally, the completely asynaptic Torres (2n=6x=60) was hypothesized to be a putative hybrid between a tetraploid *P. dilatatum* and an octoploid cytotype of *P. virgatum* with which it is sympatric and may share morphological similarities. This hypothesis, however, does not explain the lack of chromosome pairing in Torres given that variations of the IIJJ genomic formula have later been assigned to tetraploid *P. dilatatum* sexual biotypes and tetraploid *P. virgatum* (Burson et al. 1982).

An extensive program of interspecific hybridizations undertaken during the 1970s provided the foundations for the current assignment of genomic homologies within the group. Burson (1983) summarized this information including genomic formulae and putative diploid donors for the component genomes. The sexual tetraploids were assigned the IIJJ genomic formula, and they were hypothesized to have originated independently from the diploid sources for these genomes. The diploid genome donors were thought to be *P. intermedium* and *P. juergensii*, respectively (Burson 1978, 1979). Several other putative I genome donors have been identified since then (Quarín and Normann 1990, Caponio and Quarín 1993). The phylogenetic relationships among these putative genome donors have recently been shown to be complex, spanning a polyphyletic array of species (Vaio et al. 2005), and the identity of the direct donor of the

I genome has not been clarified yet. It is likely that new additional sources of the I genome will be identified in the future.

In the arrangement presented by Burson (1983), the pentaploid biotype was thought to be derived from a cross between *P. dilatatum* ssp. *flavescens* (IIJJ) and an unknown hexaploid with the IIJJXX genomic formula. A hexaploid with the appropriate meiotic behavior was described in Uruguay (Albicette 1980), and later, this hexaploid was found to possess the predicted IIJJXX genomic formula (Burson 1991). Because *Paspalum* species in general have very small genomes (Jarret et al. 1995), chromosomes are generally small and relatively featureless; as a consequence, all of the cytogenetic work relied solely on chromosome numbers and pairing in interspecific hybrids. Only recently have prometaphase karyotypes been used as a source of phylogenetic information in the genus (Speranza et al. 2003, Vaio et al. 2005). However, assessing relationships between specific fixed apomicts requires techniques that can identify individual clones as putative parents. For this approach multiple accessions of each biotype must be used to account for variability. Complete collections of all the biotypes are not currently available.

Despite the lack of a comprehensive sample of all biotypes, an effort was made to assemble a collection representing, at least partly, some of the variability within all of the biotypes. A set of microsatellite markers developed for *P. dilatatum* ssp. *flavescens* (Chapter 2) was used to describe the relationships among the apomictic biotypes of the Dilatata group, find evidence of multiple origins or variability within them, and assess their putative relationships with the sexual tetraploids. The variability and putative evolutionary patterns within the common pentaploid biotype *P. dilatatum* ssp. *dilatatum*

will be discussed in depth elsewhere; here this biotype will be represented by a few common genotypes.

Materials and Methods

Plant Material

Seeds were retrieved from the USDA Plant Introduction Station (Griffin, Georgia, USA) and the Germplasm Bank at the Facultad de Agronomía, University of the Republic, Uruguay (Table 2). Seeds of *P. dasypleurum* were kindly provided by Ing. For. R. Vergara from the Universidad Austral de Chile, and leaf material of an additional accession of *P. dilatatum* Virasoro was provided by Dr. G. H. Rua from the Universidad de Buenos Aires, Argentina. All four accessions of *P. dilatatum* Chirú come from the location indicated in Figure 4-1 and represent 4 different clusters of plants up to 2 km apart. The heptaploid clone 59B of *Paspalum dilatatum* was collected near Villa Serrana (Lavalleja, Uruguay) in 1997. The site was revisited in 2000, and the same individual was identified and recollected. Samples of both *P. dilatatum* ssp. *dilatatum* and *P. dilatatum* ssp. *flavescens* from the same site were included in this study. Silica-gel dried leaves of two triploid clones (N.A. 7663 and 7608) and a diploid individual (N.A. 7623) were used to represent *P. quadrifarium*.

Accessions were classified by biotype at the Facultad de Agronomía, Uruguay, but a preliminary biotype assignment for the USDA material was made based on field notes except for previously described accessions (mostly Burson et al. 1991). Plants were grown to the reproductive stage, and biotype assignments were corrected or confirmed by chromosome counting whenever morphology did not agree with field notes. Chromosome counts were made as described in Speranza et al. (2003). Briefly, root tips were treated with 2mM 8-hydroxyquinoline for 4 h, fixed in 3:1 (ethanol: acetic acid) for

at least 24 h and stored in 70% ethanol at 4ºC. After fixation root tips were digested in 4% (w/v) cellulase (Calbiochem) and 4% (v/v) pectinase (Sigma), squashed and stained with lacto-propionic (1:1) orcein.

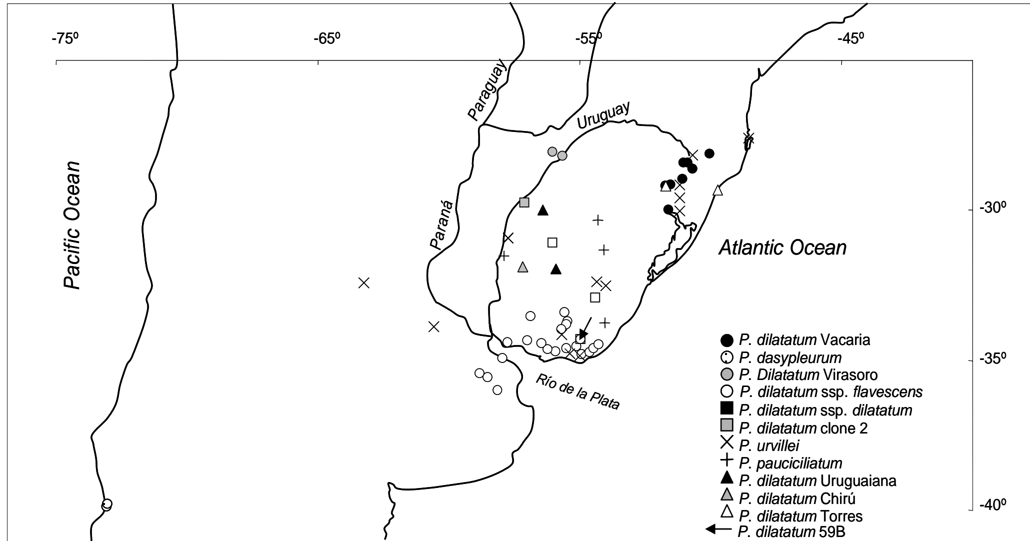

Fig. 4-1. Geographical distribution of the accessions

DNA Extraction and Microsatellite Analysis

Fresh leaves were collected and DNA extracted with Sigma Genelute^{TM} kit (Sigma-Aldrich, St. Louis, MO). Microsatellite amplification and separation were carried out as described in Chapter 2. Eleven primer pairs designed for nuclear microsatellites (Table 4-3) and chloroplast microsatellite primer pair cpDilB were chosen from those reported in Chapter 2. Microsatellite amplification, separation, and detection were performed as described in Chapter 3. Extended forward primers with an M13 tail were used in combination with labeled M13 forward primers. Three different fluorochrome labels were used (D2, D3, and D4, Beckman Coulter, Fullerton, CA). Chromatograms were visualized on CEQ TM Genetic Analysis system software (Beckman Coulter, Fullerton, CA) and scored manually.

Data Analysis

Alleles in complex patterns were assigned to loci based on the approach given in Chapter 2 for all biotypes that are known to contain a basic IIJJ genome assemblage. For *P. pauciciliatum*, assignment was preliminarily made the same way. In the case of *P. dilatatum* Torres, homology assessment was not attempted, and the data are presented only graphically by primer pair. For *P. dilatatum* Uruguaiana and *P. dilatatum* 59B, assignment was made a posteriori.

Table 4-2. Accession numbers of the materials retrieved from germplasm banks used to analyze the relationships among the different biotypes

P. dilatatum Chirú	*P. dilatatum* ssp. *flavescens*	*P. urvillei*
N.A.7537*	N.A.7493	PI 462305
N.A.7662*	N.A.7494	PI 462306
N.A.7672*	N.A.7495	PI 509008
N.A.7359*	N.A.7363	PI 509009
P. dilatatum ssp. *dilatatum*	N.A.7364	PI 509010
N.A.7673*	N.A.7433	PI 509012
N.A.7542*	N.A.7434	PI 509013
N.A.7365*	N.A.7355	PI 164065
P. dilatatum clone 2	N.A.7438	N.A.2957
PI 310044*	N.A.7441	N.A.7392
P. dilatatum clone 59B	N.A.7468	NA 7390
N.A.7686*	N.A.7470	N.A.7389
P. dilatatum Torres	N.A.7474	PI 203752
N.A.7196*	N.A.7475	N.A.7199
PI 404439**	N.A.7476	*P. dilatatum* Vacaria
P. pauciciliatum	N.A.7486	PI 404398
NA 7533*	N.A.7492	PI 404388*
PI 310222*	PI 508723	PI 404370
N.A.2500*	PI 508720	PI 404382
PI 310214*	PI 508716	PI 404372
P. dilatatum Uruguaiana	PI 508722*	PI 404434
N.A.7527*	*P. dilatatum* Virasoro	PI 508689*
PI 404444**	N.A.7207	PI 404436*

Microsatellite data were summarized using MSAnalyzer (Dieringer and Shlöterer 2002). Multilocus fixation indices (F_{IS}), genetic diversity (H_e), and population differentiation tests (Goudet et al. 1996) for the tetraploid biotypes were performed with Genepop (http://wbiomed.curtin.edu.au/genepop/).

The distribution of microsatellite variance between and within sexual biotypes was assessed with AMOVA (Excoffier et al. 1992) as perfomed by Arlequin (Schneider, Roessli, and Excoffier 2000). The same program was used to compute Slatkin's linearized distances (Slatkin 1995) to analyze the similarities among the tetraploids. For Vacaria and *P. urvillei*, Mantel tests (Mantel 1967) were performed to test isolation by distance between the geographical distance matrices and both average squared distance $(\delta\mu)^2$ (Goldstein et al. 1995) and proportion of shared alleles (D_{ps}) (Bowcock 1994). Distance matrices were produced by MSAnalyzer. Both the Mantel tests and the geographical distance matrices were obtained with Passage (Rosenberg 2001) from the geographical coordinates of accessions.

The ancestry and possible admixture of apomicts and their contributions to hybrids were analyzed using Structure (Pritchard et al. 2000). The ancestry model was set to admixture, and population information was only used as a starting point. All further simulations were performed based solely on the genotype of each individual. The number of clusters (K) was set to 5 to represent the five known tetraploids in the group. Otherwise all the default options were left in effect. Simulations were carried out for 50000 burn-in runs followed by 100,000 MCMC generations.

Results

Variability in the Tetraploids

Among the three biotypes that were best represented in this data set, *P. urvillei* was the most variable for all loci except Pdfl4 and Pdfl7, and Vacaria had the lowest F_{IS} in spite of having the lowest gene diversity (Table 4-3). These two biotypes show rather high fixation indices (0.793 and 0.734, respectively). To analyze whether at least part of the F_{IS} values found for *P. urvillei* and Vacaria can be attributed to isolation by distance,

Mantel tests were performed between genetic and geographical distance matrices for both biotypes, but no significant correlations were found (data not shown). The lowest F_{IS} over all the tetraploids was estimated for *P. dasypleurum*, although this value could be highly biased because two individuals were available. The two individuals of Virasoro were also completely homozygous. The breeding system and population structure of *P. dilatatum* ssp. *flavescens* was studied in detail elsewhere (Chapter 3) and will not be discussed here.

Samples of *P. dilatatum* ssp. *flavescens* from both Argentina and Uruguay were included in this study; however, all the alleles found in the Argentinean accessions were also present in the Uruguayan populations, and all individuals within the biotype were assigned to the same population cluster by Structure (Fig. 4-2). The five tetraploid biotypes were well differentiated based on the AMOVA, with a highly significant 89% of the variability found among biotypes (Table 4-4). The exact test of population differentiation also showed highly significant differentiation for all population pairs (not shown).

The results obtained with Structure generally assigned all individuals within each biotype to the same population cluster, showing very clear differentiation among the five tetraploid biotypes and significant admixture only for one individual in Vacaria and some components of Vacaria within *P. urvillei* (Fig. 4-2). Some admixture of *P. dasypleurum* within *P. urvillei* in one individual was also estimated. Finally, no variability for the chloroplast marker cpDilB was found within biotypes except for one individual of *P. urvillei* that showed the chloroplast haplotype found in *P. dilatatum* (Table 4-3).

Table 4-3. Summary of the microsatellite data for the sexual tetraploid biotypes of *Paspalum* group Dilatata and genotypes for the apomictic biotypes.

Biotype or Species		*P. dasypleurum*	*P. d.* ssp. *flavescens*	*P. urvillei*	*P. d.* Vacaria	*P.d.* Virasoro	*P. pauciciliatum*	*P. d.* Chirú	*P. d.* ssp. *dilatatum*	[e]*P. d.* 59B	[e]*P.d.* Uruguaiana	*P. quadrifarium*	*P. quadrifarium*
2n (x=10)		4x	4x	4x	4x	4x	4x	6x	5x	7x	6x	3x	2x
N[a]		2	21	14	8	2	4	4	3	1	2	2	1
Locus Pdfl11	H_o[b]	0	0	0.071	0	0							
	H_e[c]	0	0.070	0.690	0	0							
	A[d]	1	2	4	1	1			172	174		172	172
	Range	174	176-180	172-180	176	172	172	172	176	180	180	176	176
Pdfl4	H_o	0	0.036	0	0.125	0							
	H_e	0	0.492	0.349	0.125	0							
	A	1	4	2	2	1		196	202				
	Range	212	202-232	202-204	204-210	198	206	206	204				
Pdfl8	H_o	0	0	0.143	0.250	0							
	H_e	0	0.314	0.746	0.700	0							
	A	1	3	6	5	1		230	230				
	Range	230	236-240	198-244	218-236	230	247	234	238				
Pdfl28	H_o	0	0	0.357	0	0							
	H_e	0	0.857	0.722	0.4	0							
	A	1	10	5	2	1		188	198				
	Range	200	244-274	194-202	194-200	188	188	198	200				
Pdfl15b	H_o	0.500	0	0	0	0							
	H_e	0.500	0	0.148	0	0							
	A	2	1	2	1	1			212				
	Range	210-212	212	202-212	212	214	214	214	214				
Pdfl15	H_o	n.d.	0.036	0.154	0.125	0							
	H_e	n.d.	0.776	0.942	0.775	0.667							
	A	n.d.	8	12	5	2		234	222/224				
	Range	n.d.	216-282	244-353	243-250	228-242	-	264	226/242/250				
Pdfl22	H_o	0.500	0	0.286	0.125	0							
	H_e	0.500	0.701	0.876	0.125	0.667							
	A	2	5	11	2	2		184	186				
	Range	214-220	214-228	184-260	196-240	194-196	196	196	196				
Pdfl7	H_o	0	0	0	0.125	0							
	H_e	0	0	0	0.325	0							
	A	1	1	1	2	1	219	233	235				
	Range	241	257	251	249-251	233	231	237	257	241		241	225
Pdfl10	H_o	0	0	0.091	0.125	0							
	H_e	0.667	0.512	0.835	0.692	0							
	A	2	4	8	4	1			181				
	Range	227-237	225-229	181-229	195-227	181	179	181	203/207				
Pdfl20b	H_o	0.5	0	0	0	0							
	H_e	0.5	0.590	0.561	0	0							
	A	2	3	3	1	1	190	187	199				
	Range	199-200	197-199	196-203	199	201	201	201	201				
Pdfl20	H_o	0	0	0.077	0.125	0						208	
	H_e	0	0.543	0.594	0.758	0						206	
	A	1	3	5	4	1		219	217			187	195
	Range	215	235-239	208-263	211-273	221	225/227	223	233	187	204	204	208
Pdfl12	H_o	0	0	0	0	-							
	H_e	0	0.486	0.571	0	-							
	A	1	2	4	1	-			222				219
	Range	212	235-241	221-233	174	-	224	221	235	207		207	241
Pdfl12b	H_o	0	0	0.286	0	0							
	H_e	0	0	0.254	0	0							
	A	1	1	2	1	1	201	201	201				
	Range	201	201	201-210	201	210	210	210	210				
Pdfl18	H_o	0	0	0.214	0.125	-							
	H_e	0.667	0.491	0.807	0.125	-							
	A	2	3	7	2	-	258	258	238			224	224
	Range	264-266	236-254	252-304	254-260	-	308	274	258	230	230	230	226
cpDilB		216	217	218/217	217	217	213	217	217	217	217		
	F_{IS}	0.571	0.988	0.793	0.734	1.000							
	H_e	0.269	0.424	0.641	0.302	0.167							

[a] Sample size.

[b] Observed number of heterozygotes.

[c] Gene diversity.

[d] Number of alleles.

[e] Only the alleles not present in common *P. dilatatum* ssp. *dilatatum* are shown here. The alleles shared with *P. dilatatum* ssp. *dilatatum* are shown in Fig. 4-2.

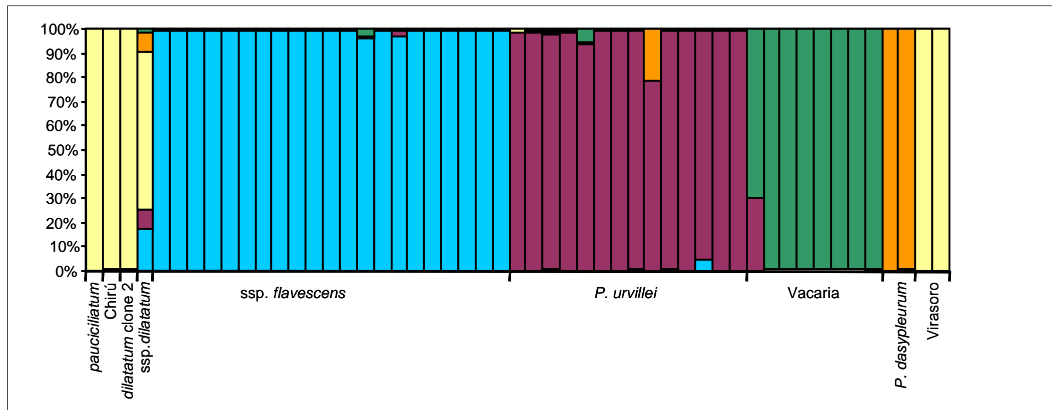

Fig. 4-2. Population structure of a sample of the members of the Dilatata group estimated by Structure under the admixture model based on microsatellite data for 13 loci. Each color represents the contribution to each genotype of each one of the five clusters generated

If the five tetraploid biotypes were derived from a single ancestral population, and given that a strong ascertainment bias could not be detected within this group (Chapter 3), Slatkin's linearized R_{ST} would represent relative divergence times between pairs of biotypes. R_{ST} distances are expected to be linearly related to evolutionary divergence time and could be used to estimate phylogenetic relationships among the biotypes. A UPGMA tree based on linearized R_{ST} is shown in Fig. 4-3a. Under this hypothesis, biotypes Vacaria and Virasoro are very similar, while *P. dasypleurum* and *P. urvillei* form a tight cluster to which *P. dilatatum* ssp. *flavescens* attaches. In this result, *P. dilatatum* as currently delimited can still be considered monophyletic with *P. dilatatum* ssp. *flavescens* very close to a sister clade formed by *P. urvillei* and *P. dasypleurum*.

To circumvent the reliance of the R_{ST}-based measures on a stepwise mutational model of microsatellites, the proportion of shared alleles (D_{ps}) was also used as a distance measure. The UPGMA phenogram based on D_{ps} shows high distance estimates for all population pairs with the nearest pair (*P. dasypleurum*-Vacaria) joined at a distance of

0.77 (Fig. 4-3b). The arrangement of the biotypes is completely different from that obtained with R_{ST}. In this case all the possible rooting options would nest either *P. dasypleurum* or *P. urvillei* within *P. dilatatum*.

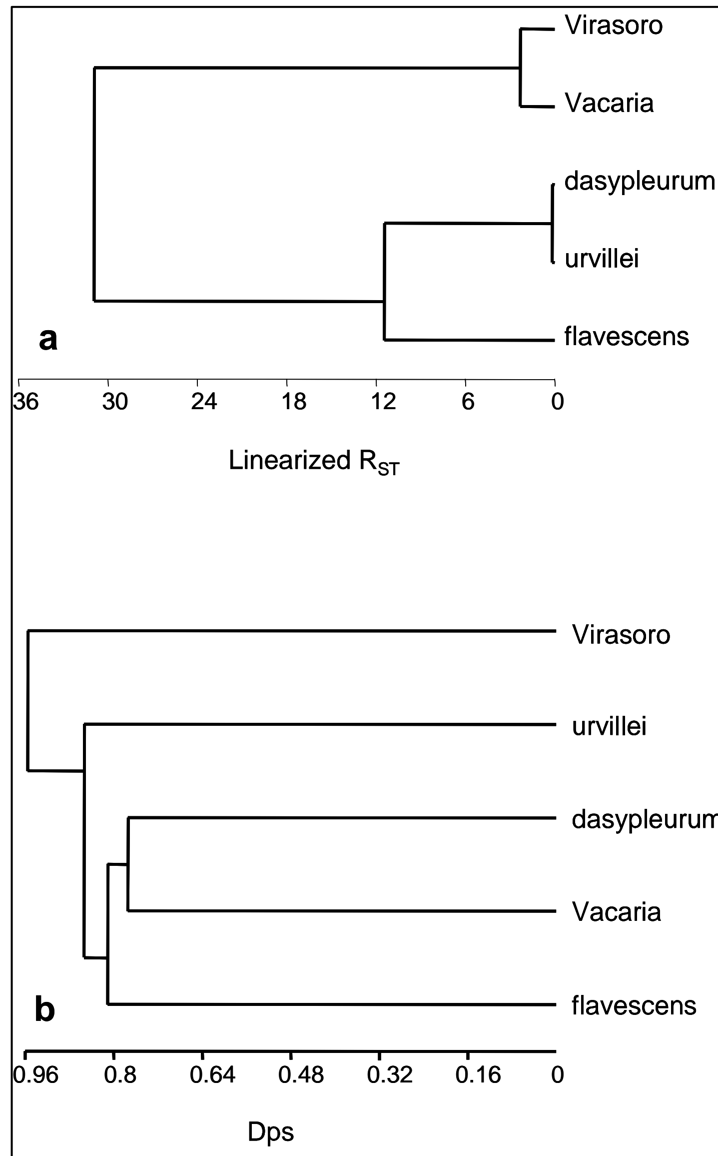

Fig. 4-3. UPGMA phenograms of the distances among the sexual tetraploid biotypes of the Dilatata group based on 13 microsatellite loci obtained with different distance measures a. Linearized R_{ST} b. Proportion of shared alleles

Variability in the Apomicts

P. pauciciliatum and *P. dilatatum* Chirú, Uruguaiana (Table 4-3), and Torres (not shown) appeared as single clones; individuals within each biotype share a number of

heterozygous allele combinations ranging from a minimum of 4 in *P. pauciciliatum* to 13 among the different accessions of *P. dilatatum* ssp. *dilatatum*. Within Torres and Chirú, no differences were detected among accessions, and the single genotypes found are shown in Fig. 4-3. In the case of pentaploid *P. dilatatum*, two clearly different clones were identified among the individuals reported here, a typical, widespread clone and a second clone referred to here as *P. dilatatum* clone 2 (Fig. 4-4). On the other hand, mostly in typical *P. dilatatum* ssp. *dilatatum* (Table 4-3) and Uruguaiana (Fig. 4-4), individuals differ by a small number of allele length differences attributable to mutation. Most of this variability is restricted to the longer alleles of loci Pdfl10 and Pdfl15 (Table 4-3 and Fig. 4-3). The highly variable longer allele in locus Pdfl15 showed a different length in each of the 3 individuals of typical pentaploid *P. dilatatum* presented here (Table 4-3). Due to its extreme instability, this locus was not used in any of the comparisons among biotypes.

Relationships among Apomicts

Paspalum. dilatatum clone 59B and Uruguaiana share 11 or 12 heterozygote allele combinations with the typical clone of *P. dilatatum* ssp. *dilatatum,* and differences are again restricted to the longer alleles of loci Pdfl10 (Fig. 4-3) and Pdfl15 (not shown). If these shared bands are interpreted to be homologous, the “extra” bands (Table 4-3) can be assumed to be located on the extra 10 chromosomes (Burson’s (1995) X_2 genome). All these bands were also found in the two triploid *P. quadrifarium* clones (Table 4-3) except for the 180-bp band in locus Pdfl11 shared by both Uruguaiana and 59B.

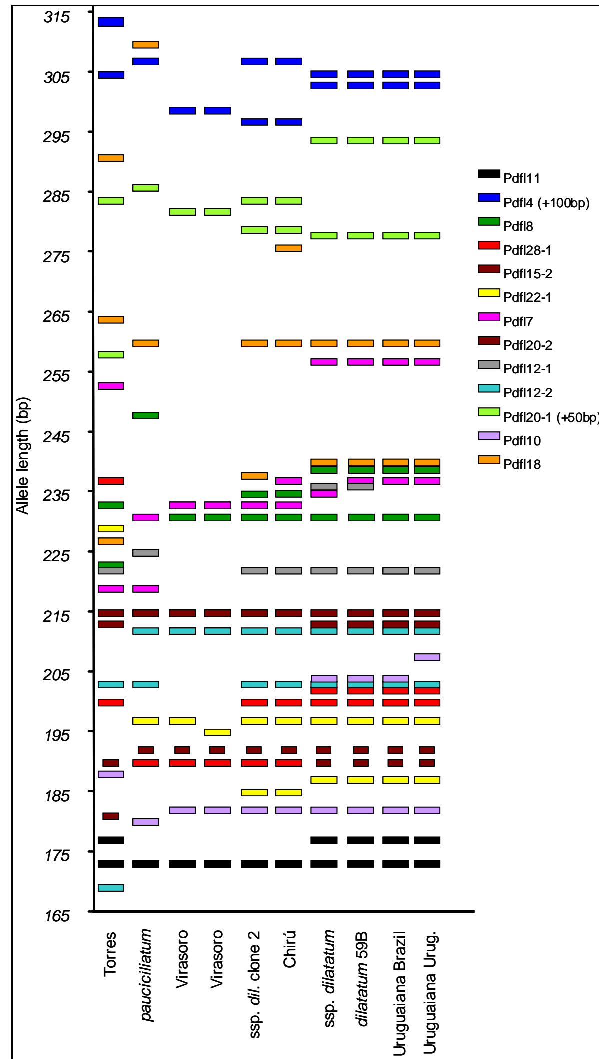

Fig. 4-4. Multilocus genotypes of the apomictic components of the Dilatata group

Chirú, *P. pauciciliatum*, and *P. dilatatum* clone 2 clearly share a significant proportion of bands with Virasoro; however, Chirú contains alleles that cannot be directly attributed to its putative Virasoro ancestry. The typical *P. dilatatum* ssp. *dilatatum*, on the other hand, appears to contain alleles that can be assigned to *P. dilatatum* ssp. *flavescens* (Fig. 4-2). The putative contributions of Virasoro, Chirú, and *P. dilatatum* clone 2 to other biotypes are confounded because they all share a significant proportion of alleles themselves (Fig. 4-2). Chirú and *P. dilatatum* clone 2 share 6 out of 8

heterozygous allele combinations making the likelihood of a sexual event linking the two very low.

All the apomicts except Torres shared a 170-bp allele in locus Pdfl28b and a 177-bp allele in locus Pdfl20c which are thought to be located on the X genome (Chapter 2). Torres does not show the 177-bp band in locus Pdfl20c and it shows a 218-bp allele in the chloroplast locus cpDilB instead of the 217-bp allele found in the other biotypes of *P. dilatatum*. Torres frequently showed more alleles in the nuclear loci than the biotypes known to be built on the basic IIJJ combination of the group. Alleles were scored to maximize its similarity to other biotypes in the data set. This approach makes sense if the asynaptic behavior of Torres is due to lack of chromosome homology and is not under genetic control. In this case, if Torres is part of the Dilatata group, it can only contain one copy of each of the I and J genomes. Even following this strategy, it is not possible to derive all of its alleles from any other apomict in the group. In spite of this, at least one allele from each of nine loci can be traced to one of the two clones of pentaploid *P. dilatatum* (Fig. 4-3).

Heterozygosity of the Apomicts

The degree of heterozygosity in the apomicts is variable. Typical *P. dilatatum* ssp. *dilatatum* is heterozygous for every locus, while Chirú and *P. dilatatum* clone 2 show lower levels of heterozygosity (Fig. 4-3). In the case of *P. pauciciliatum*, half of the loci located on an I or J genome are expected to be hemizygous because its genomic architecture does not allow for two copies of both genomes, so the observed presence of only one band at several loci cannot be reported as homozygosity as was done for Chirú and *P. dilatatum* clone 2. A similar situation is found in Torres in which only Pdfl10 amplified a single band (Fig. 4-3). In fact, based solely on its asynaptic meiotic behavior,

all the bands amplified by a single primer pair should be reported as homeologous and the genotype as completely hemizygous.

Discussion

Evolutionary relationships among the sexual tetraploid biotypes

The use of microsatellite data to assess phylogenetic relationships is questionable (Garza et al. 1995, Doyle et al. 1998). Size homoplasy, stochastic effects of past bottlenecks, allele size asymmetry, and the possibility that at least some of the biotypes had independent origins (making a hierarchical hypothesis of relatedness meaningless) are some of the concerns.

The effect of size homoplasy on population parameter estimates has been frequently discussed, and its consequences have been modeled and predicted at the populational level (Estoup 2002). Size homoplasy at the interspecific level has also long been a concern (Doyle et al. 1998). On the other hand, the use of microsatellite data for phylogeny reconstruction within species and between species has been tested against other sources of information with relative success (Alvarez et al. 2001, Richard and Thorpe 2001). In the present data set, however, it is rather the strong asymmetry in allele sizes observed among the biotypes that raises the major concerns. This problem is in some way related to the artifact known as ascertainment bias (Ellegren et al. 1995) where the non-focal species appear as less variable due to the original selection of loci to show long allele sizes in the focal species. It has been well established that the length of a microsatellite allele is related to its variability and mutation rate (Symonds and Lloyd 2000). Once a microsatellite increases its length, it also increases its upward mutation rate. One peculiarity of this microsatellite set as applied to the current group of species is that different loci seem to have been amplified in different biotypes reaching higher allele

sizes associated with higher mutation rates (Pdfl 28 in *P. dilatatum* ssp. *flavescens*, Pdfl22 in *P. urvillei*, and Pdfl20 in Vacaria) (Table 4-3). As a consequence, when applying distance measures based on allele length, the biotypes not showing long allele sizes at a particular locus necessarily appear as more similar to one another. However, this artifactual similarity is in reality a symplesiomorphy in a phylogenetic context. When applying a distance measure based only on the presence of identical alleles (D_{ps}), the larger alleles are not matched with those of other biotypes; these biotypes appear similar to one another again based shared ancestral character states. This problem is further evidenced by the high distance values among pair of biotypes obtained with D_{ps}. Alvarez et al. (2001) circumvented similar problems in *Lycopersicon* by using only loci showing very low diversity indices (<0.25). If such a criterion were applied to this data set, most information would be eliminated because the only loci showing diversity indices near 0.25 are Pdfl15b, Pdfl7 and Pdfl12b, of which Pdfl7 is null in most *P. urvillei* accessions.

The tetraploid components of the group have been previously proposed to have originated independently (Burson 1983), a possibility that is somewhat supported by the presence of fixed, non-shared chloroplast haplotypes among the three named species: *Paspalum urvillei*, *P. dasypleurum* and *P. dilatatum*. This scenario is further supported by the clear genetic differentiation in their nuclear microsatellite loci. With abundant evidence accumulating for the recurrent formation of polyploids in the last years (D. Soltis and P. Soltis 1993, P. Soltis and D. Soltis 2000), the independent formation of at least some of these biotypes remains a likely possibility. Regardless of mode of origin of

the five sexual tetraploids, the hypotheses of relatedness presented in Figure 4-2 should be interpreted with extreme caution.

On the other hand, evidence for ongoing gene flow was only found between Vacaria and *P. urvillei*. Valls and Pozzobon (1987) reported, based on field observations, that Vacaria formed natural hybrids with *P. urvillei* where their areas of distribution overlapped, while natural hybrids have not been recorded between Virasoro or *P. dilatatum* ssp. *flavescens* and *P. urvillei.* This gene flow apparently has not had strong effects on the genetic identity of the involved biotypes which remain morphologically and genetically distinct. Although more data are required to quantify any degree of gene flow among the tetraploid biotypes of *P. dilatatum* and analyze their morphological differentiation in a systematic way, it seems clear that they form distinct units and should all deserve at least formal subspecific status.

Genetic structure of the sexual tetraploids

The genetic structure of *P. dilatatum* ssp. *flavescens* has been discussed in detail elsewhere (Chapter 3). For the other biotypes, the topic has been specifically addressed only in Virasoro (Hickenbick et al. 1992). Hickenbick et al. (1992) concluded that both selfing and cross-pollination must occur in this biotype based on segregating progenies detected using isozyme markers. They also observed developing embryos in Virasoro spikelets dissected prior to anthesis. In this study, the two individuals analyzed were fully homozygous for the 13 loci, indicating a high level of homozygosity; however, very few differences were detected between the two accessions scored, making it difficult to detect the occurrence of allogamy. *Paspalum dilatatum* ssp. *flavescens* (Chapter 3) and Virasoro show relatively shorter anthers and stigmata than the other biotypes, characteristics that are usually considered morphological evidence of functional

autogamy (Frankel and Galun 1977, Campbell 1999). *Paspalum urvillei*, *P. dasypleurum* and Vacaria showed lower levels of inbreeding. Even though isolation by distance could not be demonstrated in this data set, it is likely that at least part of the observed homozygosity may be due to crossing between individuals carrying the same alleles.

Variability within the apomicts

Paspalum dilatatum ssp. *dilatatum* was the only apomictic biotype that showed evidence of containing more than a single clonal genotype. In spite of this, some microsatellite variability was found within typical *P. dilatatum* ssp. *dilatatum*, Uruguaiana and *P. pauciciliatum*. Such variability could be useful in assessing genetic diversity and phylogeographic patterns in these rather widespread clones, especially in *P. dilatatum* ssp. *dilatatum*.

The addition of the X genome, apomixis, and the origin of pentaploid *P. dilatatum*

Two different pathways have been suggested for the origin of the pentaploid IIJJX biotype. The X genome could have been added to the group by means of a hexaploid (IIJJXX) and then transferred to the pentaploids when this hexaploid crossed to a tetraploid (IIJJ) (Burson 1983, Moraes Fernandes et al. 1968) or it could have been directly added to form a pentaploid by a 2n+n hybridization between a tetraploid (IIJJ) and an unrelated diploid (XX) (Moraes Fernandes et al. 1968). The data discussed above show that the two hexaploid candidates identified so far are more likely explained as derivatives than progenitors of the pentaploids. The second pathway invokes the production of unreduced gametes by *P. dilatatum* tetraploids. Chloroplast sequence data support the derivation of the maternal genome from within *P. dilatatum* rather than from the donor of the X genome (Chapter 2). Production of unreduced gametes by tetraploids has been observed (Speranza unpub. res.). One *P. dilatatum* ssp. *flavescens* individual,

when pollinated by Chirú produced several 2n+n (2n=7x=70) hybrids. The hybrids of the same *P. dilatatum* ssp. *flavescens* individual by Virasoro (Chapter 2), when crossed with Chirú also produced several 2n+n hybrids with 2n=7x=70. This time, the maternal plant was expected to be highly heterozygous and its genotype can be inferred from the segregation data shown in Chapter 2 for its progeny. Analysis of its 2n+n hybrids with Chirú, however, did not show the transmission of any heterozygosity from its maternal parent for several microsatellite loci (data not shown). This indicates that the unreduced gametes were produced by some second division restitution (SDR)-like mechanism. Even if the possibility cannot be rejected, no mechanism has been observed that can explain the formation of a primary pentaploid by the contribution of a heterozygous unreduced gamete by a heterozygous tetraploid. There are, however, in the present data set, relatively homozygous pentaploids, namely *P. dilatatum* Clone 2. The level of heterozygosity in the pentaploids is then variable. How did a heterozygous pentaploid form if hexaploids are excluded as intermediaries? Existing pentaploid individuals have the ability to transmit euploid IJX gametes and produce new IIJJX pentaploids when crossed to an IIJJ tetraploid (Mazzella and Speranza 1997). The observed extreme heterozygosity could have been gained by existing pentaploids by successive cycles of crossing to other tetraploid members of the group. An extensive survey of the intrabiotypic variability within *P. dilatatum* could provide further evidence of this mechanism.

***Paspalum dilatatum* Uruguaiana and 59B**

Both Uruguaiana and 59B share almost all of the heterozygous allele combinations found in the widespread typical *P. dilatatum* ssp. *dilatatum*. As a consequence, their mutual relationship and the relationship of both clones to the pentaploid biotype cannot

be explained by sexual events. Early pollination of pentaploid florets has been shown lead to the fertilization of aposporic embryo sacs and the production of 2n+n hybrids (Bennett et al. 1969, Espinoza and Quarín 2000, Burson 1992). Furthermore, a putative donor of the alleles not present in the pentaploid parent has been identified here as *P. quadrifarium*. This suggests that these two clones are 2n+n hybrids of pentaploid *P. dilatatum* and a diploid or tetraploid individual of *P. quadrifarium*. The close morphological and physiological similarity between Uruguaiana and pentaploid *P. dilatatum* has been previously noted (Burson 1991). Furthermore, the spikelets of Uruguaiana (and clone 59B) differ from those of the pentaploid biotype in that their maximum width is located near the middle of the spikelet like those found in species of the Quadrifaria group (Barreto 1966) rather than near the base as seen in *P. dilatatum*. The two accessions of Uruguaiana analyzed here were collected more than 220 km apart, and they differ at some microsatellite loci. These differences were interpreted as mutations given that the alleles hypothesized to represent the X_2 genome were identical for the two locations. Its geographical range and the presence of mutations suggest that Uruguaiana is not a recent derivative of pentaploid *P. dilatatum* and that several more such hybrids could have been formed in a similar way. The finding of 59B, a single hybrid of the same kind shows that the complex is active and still generating new combinations at higher ploidy levels. Clone 59B is a single, highly sterile individual found near Villa Serrana, Uruguay and it is likely to be the product of a contemporary hybridization event. This plant was collected at the edge of a water stream that crosses a secondary road. Clone 59B was growing among plants of *P. dilatatum* ssp. *flavescens* and pentaploid *P. dilatatum*. Immediately next to 59B were several plants of *P. exaltatum*

which shares genomes with *P. quadrifarium* (Vaio et al. 2005) and several dispersed individuals of *P. quadrifarium*. Spikelet morphology and the presence of *P. quadrifarium*-specific bands in 59B strongly suggest that a species from the Quadrifaria group could have contributed the extra genomes in 59B.

***Paspalum dilatatum* clone 2**

This accession of *P. dilatatum* can be partially explained by a cross between Virasoro and Chirú. Morphologically this accession seems to correspond to the putative Virasoro x Uruguaiana hybrids reported by Machado et al. (2005). Even though Uruguaiana is presently known to occur near the collection site of *P. dilatatum* clone 2, its genotype does not support such parentage. On the other hand, Chirú is only known from a relict population in Paysandú, Uruguay and Clone 2 was found in Brazil, approximately 240 km NE of this location. A greater distribution range of Chirú in the past or the still undetected occurrence of genotypes closely related to it in southern Brazil must be invoked to explain this hybrid as the product of a tetraploid x hexaploid hybrid. The reverse hypothesis where Chirú is a derivative of *P. dilatatum* clone 2 is considered below.

***Paspalum dilatatum* Chirú**

Chirú is closely related to Virasoro and less heterozygous than *P. dilatatum* ssp. *dilatatum* and Uruguaiana. The involvement of Virasoro as the donor of the basic IIJJ genomes seems evident; however, Chirú also shows alleles that cannot be explained by the genotypes found in Virasoro, and given that Virasoro has been found to be highly homozygous, it is not likely that the relationship between the two biotypes is direct. The reverse pathway, i.e., the addition of an extra copy of the X genome to a *P. dilatatum* clone 2 type individual in a fashion similar to that described for Uruguaiana, cannot be

excluded as the origin of Chirú. In fact, only the shorter allele in Pdfl18 and the absence of a second allele in locus Pdfl12 could not be explained this way. Under this scenario, *P. dilatatum* clone 2 would be more widespread than detected here and Chirú would not be a relict population of a formerly widespread biotype but a possibly recent 2n+n hybrid of *P. dilatatum* clone 2. The only extra putative X genome allele found in Chirú is a 187 bp allele at locus Pdfl20. This allele was also found in clone 59B, and it was attributed to its *P. quadrifarium* parent. In the case of Chirú, this constitutes very weak evidence of the origin of the X genome from within the Quadrifaria group.

Paspalum pauciciliatum

P. pauciciliatum appears to be very closely related to and is sympatric with both Chirú and *P. dilatatum* clone 2. The contribution of a reduced IJX gamete from either *P. dilatatum* biotype as a pollen donor could explain most of the alleles found in *P. pauciciliatum* and even more so if one-step mutations are allowed. Its chloroplast haplotype, however, is different from that of the other members of the Dilatata group. This difference was detected only at the cpDilB locus whereas the chloroplast haplotype of *P. pauciciliatum* is identical to that of the rest of the Dilatata group five non-coding regions (Chapter 2). Its cytogenetic architecture (Moraes Fernandes et al.1968) implies that one of its genomes is present in two copies. If the difference found in the chloroplast genome is taken as evidence of an independent origin of its chloroplast genome, then its maternal progenitor must be closely related to the diploid donor of either the I or J genome to the Dilatata group but not the same individual or population. The maternal progenitor should be responsible for the contribution of extra bands at loci Pdfl18, Pdfl12b and Pdfl7. Under this scenario, its genomic formula should be either IIJX or IJJX.

***Paspalum dilatatum* Torres**

Chloroplast DNA data strongly support the contribution of the maternal genome of Torres from within the Dilatata group (Chapter 2). However, its chloroplast haplotype corresponds to *P. urvillei* instead of that shared by all the other biotypes of *P. dilatatum*. For 7 out of 13 loci analyzed Torres shows alleles also found within either the *P. dilatatum* ssp. *dilatatum*-Uruguaiana complex or the Chirú-*P. dilatatum* clone 2 complex; however, it lacks one of the markers of the X genome that is shared by all the other apomicts including *P. pauciciliatum*. Its link to the rest of the group may be some genotype of the highly variable *P. urvillei*. If Torres contains a copy of the I and the J genomes, as suggested by its similarities to *P. urvillei*, then its other genomes must not come from another member of the group based on the lack of pairing of its chromosomes. The relationship of this biotype to the rest of the group cannot be completely clarified based on this data set and the task would probably require analyzing species belonging to other taxonomic groups.

Conclusions

This study has established a series of evolutionary hypotheses within the Dilatata group which will dramatically change the future direction of both basic and applied research within this group.

1. Both nuclear and chloroplast markers show that the five sexual tetraploids studied here are well differentiated and do not show significant gene flow except for *P. urvillei* and Vacaria.

2. The Uruguayan and Argentinean accessions of *P. dilatatum* ssp. *flavescens* form a single coherent unit.

3. *P. dilatatum* ssp. *flavescens* and Virasoro appear highly homozygous, in agreement with floret morphology and previous reports. *P. urvillei*, Vacaria and *P. dasypleurum* probably show higher degrees of allogamy.

4. No clear evidence was found to support the monophyly of the group.

5. The relationships among the sexual tetraploid biotypes could not be reliably explained by the current data set.

6. Chloroplast haplotype differentiation is in agreement with current species delimitations.

7. Uruguaiana, Chirú, Torres and *P. pauciciliatum* each appears to comprise a single clone.

8. Pentaploid *P. dilatatum* comprises an assemblage of more than one clone.

9. Variability attributed to somatic mutations was found within Uruguaiana, *P. pauciciliatum* and the typical clone of *P. dilatatum* for at least two loci, which can be used for analyzing genetic structure within these clonal biotypes.

10. All of the apomicts in the group show the same alleles in the loci thought to reside on the X genome, suggesting a single origin for this genome in the group.

11. All of the apomicts seem to contain at least one copy of each of the I, J and X genomes.

12. The hexaploids and heptaploids are better explained as derivatives of the pentaploid *P. dilatatum* rather than its ancestors.

13. Virasoro is the sexual tetraploid that shows the greatest degree of similarity to all of the apomicts.

14. A genotype of pentaploid *P. dilatatum* derived from Virasoro might have been the primary apomict in the group. Further pentaploid-tetraploid crosses may have created the variability and the heterozygosity found in the apomicts.

15. The suggested mechanisms for the formation of new apomicts involve either an unreduced female gamete or a euploid IJX pollen grain of pentaploid *P. dilatatum*.

CHAPTER 5
PENTAPLOID X TETRAPLOID HYBRIDIZATION CYCLES IN *Paspalum dilatatum* (POACEAE): EXPLAINING THE CURRENT AND FUTURE EVOLUTIONARY SUCCESS OF AN IMBALANCED POLYPLOID

Introduction

Paspalum dilatatum Poir. is a warm-season grass native to the grasslands of temperate South America. This species is part of an informal taxonomic group within the genus known as the Dilatata group (Chase 1929). Included in this group are several sexual and apomictic biotypes and species whose evolutionary relationships have recently been discussed in detail based on data from 13 microsatellite loci (Chapter 4). The sexual members of the group (*P. urvillei* Steud., *P. dasypleurum* Kunze ex Desv., *P. dilatatum* ssp. *flavescens* Roseng. Arr. et Izag., and biotypes Virasoro and Vacaria of *P. dilatatum*) are all tetraploid and share the same genomic formula (IIJJ). The relationships among the sexual tetraploids and between them and the apomicts have not been completely clarified; however, microsatellite data suggest that Virasoro has greatly contributed to the genetic makeup of the apomictic components (Chapter 4). Except for pentaploid *P. dilatatum*, the rest of the apomicts in the group (*P. dilatatum* biotypes Uruguaiana, Chirú and Torres (6x) and *P. pauciciliatum* (Parodi) Herter (4x)) seem to each comprise a single clone and its mutational derivatives, and all of them have been hypothesized to be derivatives of the pentaploid form (Chapter 4). In this context, the apomicts would all include at least one copy of the I and J genomes and at least one copy of the third unassigned genome (X). The pentaploid form (IIJJX) had previously been proposed to be the product of a cross between a hexaploid (IIJJXX) and a tetraploid (IIJJ) (Burson 1983); however, the

transmission of an unaltered heterozygous multilocus genotype between pentaploids and the hexaploids Chirú and Uruguaiana suggests that the latter may be more likely derivatives of the pentaploid form by means of a 2n+n hybridization in which the pentaploid contributed an unreduced gamete and the second X genome was contributed by a diploid. Chloroplast sequence data suggest that the pentaploid probably acted as the maternal progenitor in such crosses (Chapter 2, Chapter 4), a mechanism that had already been reported experimentally (Bennett et al. 1969, Burson 1997). Genotypic information for thirteen microsatellite loci suggests yet another mechanism by which the pentaploid may be involved in the origin of the remaining apomicts, i.e., *P. pauciciliatum*, and at least one recombinant pentaploid clone. The pentaploids are able to produce euploid IJX pollen grains (Mazzella and Speranza 1995) which can fertilize a sexual tetraploid to yield a recombinant pentaploid. Pentaploid *P. dilatatum* is not only the most widespread biotype, but it also seems to have been the basis of the entire apomictic complex in the group. It may have been involved in the origin of the other apomicts either by means of unreduced female gametes or euploid IJX pollen grains, and it could be the original carrier of the X genome. If the transmission of euploid IJX gametes is a frequent event, then several more recombinant pentaploids like the one reported in Chapter 4 should be found in the wild. Variability has been detected among pentaploid *P. dilatatum* accessions with dominant markers (Casa et al. 2002); however, with this kind of markers, and particularly for a small sample, clonal variants cannot be distinguished from recombinants. Microsatellites have proven efficient in detecting recent hybridization events and if recombinants are found it should be possible to infer their mode of origin from their genotypes.

Regardless of its mode of origin or evolutionary role, pentaploid *P. dilatatum* is currently distributed worldwide in warm-temperate regions of the north and south hemispheres where it has become an important forage grass (Burson 1983, Skerman and Riveros 1992). Because of this, there is great applied potential for the detection of genetic variability in the available germplasm collections. Microsatellite markers can reveal mutational variation that could be useful for assessing the extent and distribution of mutational genetic variability within the typical form of this biotype (Chapter 2, Chapter 4).

In this study, an extensive collection of pentaploid *P. dilatatum* representing its worldwide distribution has been retrieved from existing germplasm banks and analyzed using microsatellite markers developed for *P. dilatatum* ssp. *flavescens* (Chapter 2) to assess the level of variability.

Materials and Methods

Plant Material

Seeds of pentaploid *P. dilatatum* were retrieved from the USDA Plant Introduction Station (Griffin, Georgia) and the Germplasm Bank at the Facultad de Agronomía, University of the Republic, Uruguay. Accession numbers are given in Table 5-1. Additional dry leaves from California, Texas, and Australia were kindly provided by M. McMahon, J. Tate, and K. Smith, respectively.

Material retrieved from Georgia mostly contained populational samples. The Uruguayan collection used in this study was mostly developed by myself between 1992 and 1999. This collection is composed primarily of single-plant progenies produced in the greenhouse from off-type individuals collected in the field. Populations 1 and 3 had been previously reported by Prof. J.C. Millot (1997 pers. comm.) as variable based on

morphological evidence. These populations were collected as single individuals, and each apomictic progeny kept under a different accession number (Table 5-1). Chromosome numbers were determined for all the Uruguayan single-plant accessions by P. Speranza, M. Vaio and C. Mazzella following the technique described in Speranza et al. (2003).

For the current study, full seeds were germinated in Petri dishes on filter paper. Germinators were placed at 4ºC for 4 days prior to incubation to break dormancy and homogenize germination and were then transferred to an incubator with alternating temperatures (16 h at 30ºC light, 8 h at 20ºC dark). For eight populational samples with good seed quality, eight individuals were grown per accession, otherwise only one individual was kept in each case (Table 5-1). Plants were cultivated in greenhouse conditions for at least one complete growth season and screened for contaminants or biotype assignment errors at the reproductive stage. Chromosome numbers were determined when the originally reported biotype assignment and morphological appearance of a plant were in disagreement. Chromosome numbers were also determined *a posteriori* for all putatively recombinant individuals (Table 5-1).

DNA Extraction and Microsatellite Analysis

Fresh leaves were collected in the greenhouse and DNA extracted with Sigma Genelute™ kit (Sigma-Aldrich, St. Louis, MO). Microsatellite amplification and separation were carried out as described in Chapter 2. Eight primer pairs reported in Chapter 2 were used in this study (Pdfl4, Pdfl7, Pdfl8, Pdfl10, Pdfl11, Pdfl12, Pdfl15, and Pdfl20). Fragment amplification was obtained for all individuals by varying the amount of DNA added to the PCR mix between 0.5-3 µL. Extended forward primers with an M13 tail were used in combination with labeled M13 forward primers. Three different fluorochrome labels were used (D2, D3 and D4, Beckman Coulter, Fullerton, CA). PCR

products labeled with different dyes were combined, and 1 µL of the combined products was loaded on a CEQ 8000 capillary sequencer (Beckman-Coulter, Fullerton, CA). Chromatograms were visualized on CEQ ™ Genetic Analysis system software (Beckman Coulter, Fullerton, CA) and scored manually.

Table 5-1. Accession numbers, genotypes, and population of origin of the pentaploid *P. dilatatum* material retrieved from germplasm banks. The number of individuals analyzed per accession is indicated in parenthesis if it is more than one. The genotypes/individuals for which the chromosome number was confirmed or is first reported in this study are marked by an asterisk. A population number is given in those cases in which more than one individual from the same location was analyzed.

Accession	Genotypes	Population	Accession	Genotypes	Accession	Genotypes	Population
N.A.7346 (8)	A/RecC2*/RecC7*	7	PI 173004	M1	PI 410284	P1	
N.A.7368 (8)	P/V	2	PI 202298	G	PI 410286	A	
N.A.7404	A*		PI 202300	A	PI 410287	A	
N.A.7416	L*		PI 217623	A	PI 462248	P	
N.A.7430	Recb1*		PI 222812	A1	PI 462254 (8)	A/Q	9
N.A.7440 (8)	J/P/RecB4*	6	PI 235068	A	PI 462256 (8)	P/T	4
N.A.7465 (4)	I/P/RecB3*	1	PI 271592	A	PI 462258	A	
N.A.7471	O*		PI 273255	A	PI 462261 (8)	RecC2*	10
N.A.7524	A*		PI 274081	A	PI 462262	P	
N.A.7525	W*		PI 283015	RecA2*	PI 462264	A	
N.A.7528	D*		PI 285302	A	PI 508671	U	
N.A.7529	Y*	3	PI 300076	A	PI 508676	P	
N.A.7540	G*		PI 300077	A	PI 508682	P	
N.A.7541	P*		PI 304015	E*	PI 508692	F	
N.A.7542	A*	7	PI 310044	RecA1*	PI 508694	D	
N.A.7543	RecB2*		PI 310076	RecC4*	PI 508701	A	
N.A.7544	A*		PI 310077	N	PI 508703	D	
N.A.7545	P*		PI 310078	P	PI 508705	A	
N.A.7562 (8)	H*	8	PI 310083	D	PI 508706	A	
N.A.7563 (8)	C/RecC1*	5	PI 310088	A	PI 508707	M	
N.A.7588	I*		PI 310091	M	PI 508708	A	
N.A.7606	P*		PI 331112	A	PI 508712	B	
N.A.7609	RecC3*	3	PI 338660	A	PI 508715	A*	
N.A.7613	A*		PI 404394	P	PI 508719	G	
N.A.7618	RecC6*		PI 404410	T	PI 508725	P	
N.A.7619	H*		PI 404412	P	PI 508857	T*	
N.A.7657	X*	1	PI 404415	RecC5*	PI 576135	M	
N.A.7658	P*	1	PI 404431	I			
N.A.7661	A*	1	PI 404432	P			
N.A.7665	P*	3	PI 404823	P			
N.A.7673	H*		PI 409854	A			
N.A.7674	RecA3*		PI 410281	A			
N.A.7688	S*	1	PI 410283	A			
N.A.7690	A*		NSL 28721	D1			

Table 5-2. Allele frequency distributions in the tetraploid biotypes of *P. dilatatum* used to estimate possible contributions to the recombinant pentaploid clones.

Locus	Allele	*P. dasypleurum*	*P. dilatatum* ssp. *flavescens*	*P. urvillei*	*P. dilatatum* Vacaria	*P. dilatatum* Virasoro
Pdfl11	172			0.18		1.00
	174	1.00				
	176		0.04	0.18	1.00	
	178			0.50		
	180		0.96	0.14		
Pdfl4	196					1.00
	200					
	202		0.13	0.21		
	204			0.79	0.06	
	206					
	208					
	210				0.94	
	212	1.00				
	220		0.77			
	226					
	232		0.11			
	238					
	240					
	242					
Pdfl8	198			0.04		
	218			0.11	0.44	
	220				0.06	
	224				0.38	
	226			0.07	0.06	
	230	1.00				1.00
	232					
	234					
	236		0.11	0.32	0.06	
	238		0.82	0.39		
	240		0.07			
	244			0.07		
	266					
	272					
	274					
	276					
Pdfl15b	202			0.92		
	210	0.25				
	212	0.75	1.00	0.08	1.00	
	214					1.00
Pdfl15	216		0.04			
	218					
	222					
	224			0.08		
	226					
	228					0.50
	232					
	236		0.18			
	238		0.34			
	240					
	242		0.02		0.13	0.50
	244				0.56	
	246		0.04		0.19	
	250				0.13	
	252					
	254			0.12		
	264					
	276		0.29			
	278		0.07			
	282		0.04			
	286			0.04		
	292			0.08		
	294			0.08		
	296			0.12		
	298			0.15		
	302			0.08		
	312			0.08		
	326			0.08		
	332			0.08		
	354			0.04		
Pdfl10	175					
	179					
	181			0.18		1.00
	187			0.05		
	191			0.45		
	195			0.09	0.06	
	207					
	209			0.05		
	211				0.25	
	213				0.50	
	221			0.09		
	225		0.04			
	227	0.50	0.68		0.19	
	229		0.29	0.09		
	237	0.50				
	245					
Pdfl20	207			0.08		
	209			0.62		
	211			0.27	0.13	
	215	1.00				
	217					
	221					1.00
	223					
	233					
	235		0.50			
	237		0.04			
	239		0.46			
	263			0.04	0.31	
	271				0.19	
	273				0.38	
Pdfl12	174				1.00	
	212	1.00				
	221			0.64		
	223					
	225			0.09		
	229			0.09		
	233			0.18		
	235		0.39			
	239					
	241		0.61			
	245					
	251					
	253					
Pdfl12b	200	1.00	1.00	0.86	1.00	
	210			0.14		1.00
Pdfl7	233					1.00
	235					
	241	1.00				
	249				0.81	
	251			1.00	0.19	
	253					
	257		1.00			
	259					
Pdfl20b	196			0.36		
	197		0.25	0.57		
	198		0.57			
	199	0.75	0.18		1.00	
	200	0.25				
	201					1.00
	203			0.07		

Data Analysis

Alleles in complex patterns were assigned to loci based on Chapter 2. MSAnalyzer (Dieringer and Shlöterer 2002) was used to summarize microsatellite data and calculate distance matrices based on the proportion of shared alleles (D_{ps}) (Bowcock 1994).

The number of mutational steps of each genotype from the most common genotypes was estimated based on a D_{ps} matrix and used to decide which genotypes should be further analyzed as recombinants.

For the genotypes identified as mutational variants of the major clone, a genotype network was created using Network (www.fluxus-engineering.com) by the Median Joining method (Bandelt et al. 1999). Alleles of loci interpreted to be located on the I or J genome according to Chapter 2 were assigned to either homologous locus in each individual based on size and/or conservation of the other allele, i.e. the shorter band could be considered a mutant of the "long" allele if the common "short" allele was conserved. The entire genotype was further treated as a single haplotype because it was assumed that recombination did not take place.

Information about allele frequencies in the tetraploid biotypes of the Dilatata group was obtained from Chapter 4 and is here summarized and shown in Table 5-2 for comparison. Two exploratory approaches were followed to identify the putative donor of alleles to each recombinant pentaploid. First, a matrix of D_{ps} was generated with MSAnalyzer and the distance of each recombinant to each tetraploid biotype was taken as an indication of putative ancestry. Second, allele admixture proportions were explored using Structure (Pritchard et al. 2000). To circumvent the confounding effect of the high number of alleles shared by the typical pentaploid genotype and Virasoro, a population of a hypothetical ancestor of the typical clone was simulated by removing the alleles clearly

attributable to a Virasoro-like parent. The ancestry model was based on prior population assignments for the tetraploids and admixture for the recombinant genotypes. The simulation was re-run with the number of clusters set to represent the actual number of putative ancestral populations present in the matrix. Otherwise, all of the default options were left in effect. The final simulations were carried out for 50000 burn in runs followed by 100000 MCMC replicates.

Results

A total of 29 multilocus genotypes for 12 loci were identified using the eight microsatellite primer pairs. Primer pairs Pdfl15 and Pdfl12 amplify two and Pdfl20 three independent loci (Chapter 2). The 177-bp band which is thought to reside on the putative X genome locus Pdfl20c was present in all the individuals and is not shown in the figures. The data were first displayed graphically (as in Fig. 5-4) and visually analyzed. A single most common genotype with a few mutations was evident while a few individuals were clearly recombinants. For exploratory purposes the most frequent genotypes (A and P) were treated as ancestral and mutational steps counted from them. To assess the extent of the major clone, the number of allele size differences of each genotype to the closest of either of the two versions of the major clone was calculated (Fig. 5-1). All genotypes with 5 or fewer allele differences to the common genotype were tentatively considered its mutational variants. A highly contrasting pattern was observed between those genotypes with 7 or more allele differences with the common genotypes and those with 5 or fewer. A progression in the number of allele-size changes from 0 to 5 was clearly interpretable as somatic mutations because alleles accumulate in the most unstable loci (Pdfl15 and Pdfl10) and the rest of the loci typically remain unchanged or show single-step mutations in the longer alleles. These genotypes maintain

at least 6 original heterozygous combinations of the common genotype. This pattern is not observed in genotypes showing 7 or more allele differences from the common genotype; changes affect either allele and their size ranges are drastically different.

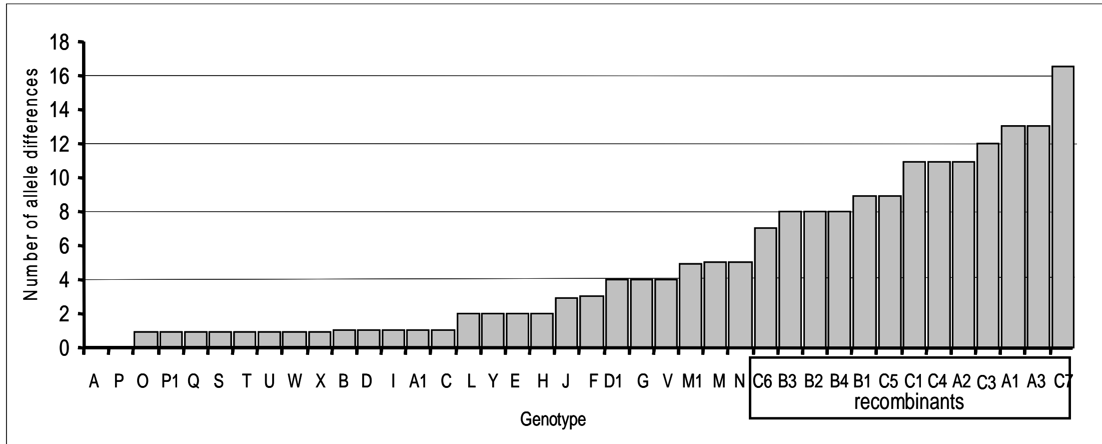

Fig. 5-1. Number of microsatellite allele differences between all genotypes of pentaploid *P. dilatatum* and the nearest of the two most widespread genotypes (A and P). The total number of alleles considered is 22.

A genotype network was built for the major clone and its inferred somatic variants (Fig. 5-2a). The network represents a completely hierarchical arrangement with a total of 44 changes in 12 variable alleles of which 13 changes in the same 4 alleles are homoplasious (long alleles in Pdfl10, Pdfl15 and Pdfl8 and the short allele in Pdfl7) (Fig. 5-2a). Two frequent genotypes (A and P) include most of the individuals. Several genotypes are connected to these two common ones by one or a few mutations. Of the total 44 changes required to build the network, six were allele losses where the actual size of the resulting allele cannot be observed. For the remaining 38 changes, 20 or 21 represent size increases and 18 or 17 size decreases depending on whether genotype A or P is considered ancestral. These changes are not distributed evenly along the network or across loci. A single branch connecting genotypes G, H, and J to A accounts for 11 size increases. The ratio of increases to decreases for the rest of the tree is 9:16. Of the 44

total changes scored for 22 alleles, 10 are accounted for by the longer allele of Pdfl15 alone and 7 by the longer allele of Pdfl10. A significant size difference of 7 steps separates a completely inactive Pdfl15 long allele of approximately 28 GT repeats (branches departing from P) from a hypermutable Pdfl15 whose size increased independently twice: once leading to genotypes M and M1 and a second time on the branch ending in H where it reaches a maximum length of approximately 40 repeats.

The geographical locations of the genotypes shown in Fig. 5-2a are displayed in Fig. 5-2b. The genotypes show a high degree of admixture in the native range of the species. A meaningful statistical treatment of the geographical structure is not possible because the sampling strategy was deliberately biased in Uruguay where most of the samples and genotypes were found. Despite this, the most remarkable geographical patterns are the absence of genotype P towards the southwest and the high degree of admixture east of the Uruguay River. This extreme admixture of genotypes is best exemplified by the presence of highly divergent genotypes within a single population (see legend of Fig. 5-2b and Table 5-1).

In contrast to the high diversity and admixture found within the native range, the samples retrieved from the rest of the world all show the A genotype except for one accession from South Africa which shows the P genotype, and the samples from Florida, Greece, and Turkey, which show the M or a related genotype (Fig. 5-2c).

As a first approach to analyze the origin of the recombinant genotypes, their allelic constitutions were compared to those of the known sexual biotypes in the group. The estimation of the putative contributions of Virasoro, Vacaria, and *P. dilatatum* ssp. *flavescens* to the recombinants suggests the same pattern when estimated by Structure

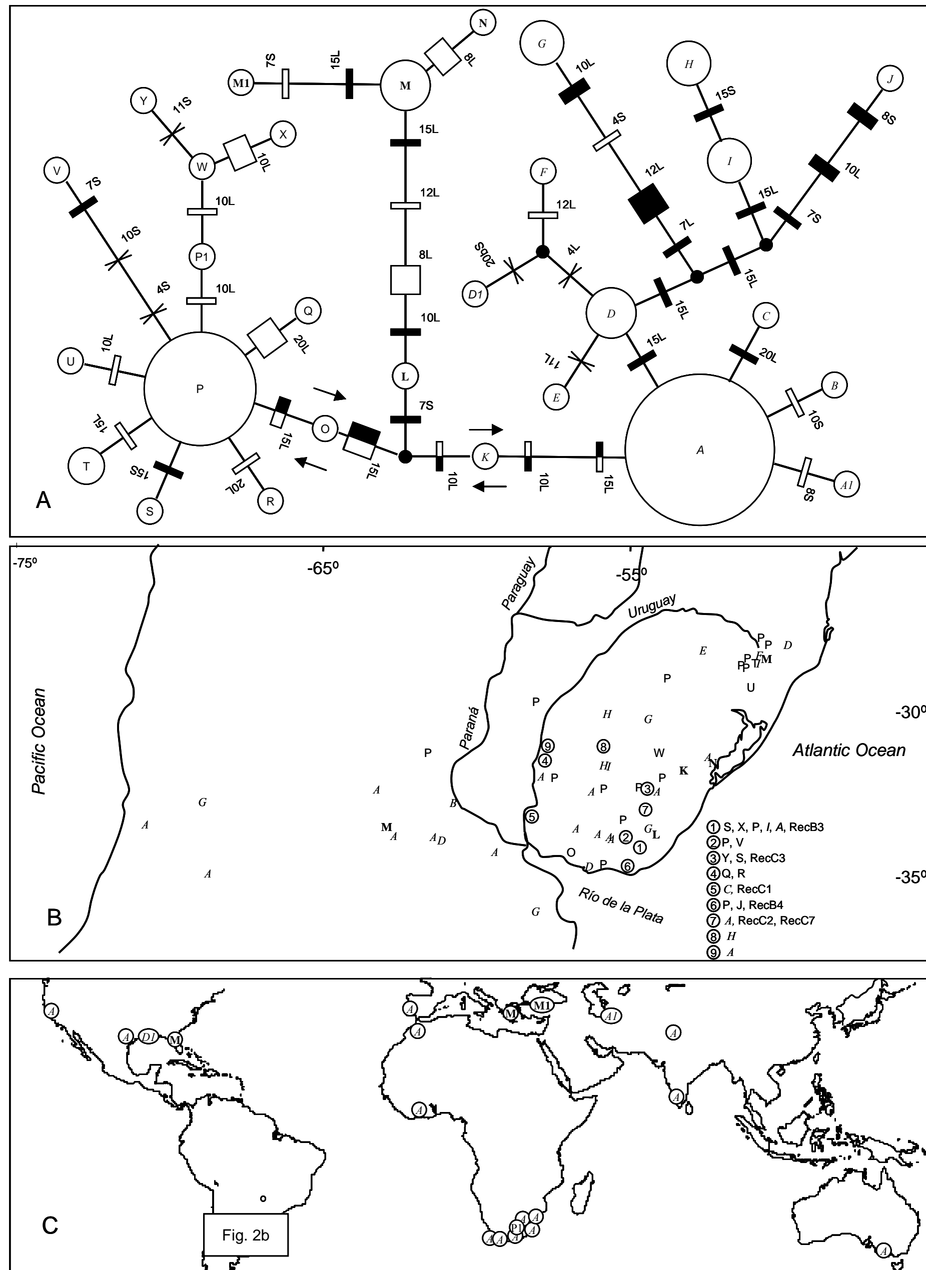

Fig. 5-2. Genotypic relationships and geographical distribution of the clonal variants of pentaploid *P. dilatatum* A. A haplotype network of the multilocus microsatellite genotypes. Allele losses are indicated by crosses and size variations as boxes on the branches assuming either genotype *A* or P is ancestral. Box sizes are proportional to allele-size increase (black boxes) or decrease (white boxes) in dinucleotide repeats. B and C.. Distribution of clonal variants of typical *P. dilatatum* B. within its native range and C. outside its native range. Letters refer to the genotypes shown in Figure. 2A. Circled numbers in Fig. 2B correspond to locations for which more than one individual was analyzed. The genotypes found in each population are indicated in the legend.

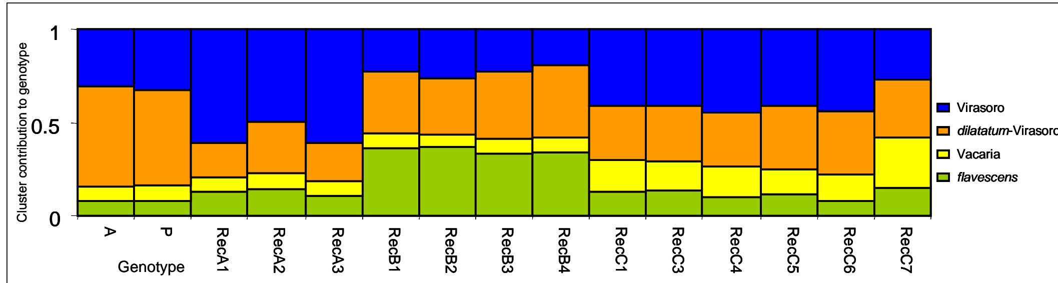

Fig. 5-3. Estimated tetraploid biotype contributions to the pentaploid recombinant *P. dilatatum* genotypes. The clusters were generated by Structure based on prior population assignments and the number of clusters (K) set to 4 to represent each of the indicated putative tetraploid sources: Virasoro, Vacaria, ssp. *flavescens*, and a fourth sample inferred from the alleles present in the most common genotypes of typical *P. dilatatum* not shared with Virasoro. The simulation resulted in no admixture estimated for the tetraploid biotypes each of which was completely assigned to its own cluster (not shown).

Table 5-3. Proportion of shared allele distances (D_{ps}) of the recombinant pentaploids of *P. dilatatum* to the tetraploid biotypes of the Dilatata group. The distance of each recombinant to the nearest biotype is indicated in bold italics.

Genotype	*P .dasypleurum*	*P. dilatatum* ssp. *flavescens*	*P. urvillei*	*P. dilatatum* Vacaria	*P. dilatatum* Virasoro
RecA1	0.90	0.94	0.85	0.95	***0.35***
RecA2	0.95	0.89	0.82	0.95	***0.50***
RecA3	0.90	0.92	0.86	0.95	***0.35***
RecB1	0.78	***0.51***	0.79	0.80	0.80
RecB2	0.83	***0.48***	0.79	0.86	0.75
RecB3	0.85	***0.55***	0.78	0.86	0.80
RecB4	0.78	***0.55***	0.78	0.80	0.85
RecC1	0.85	0.86	0.78	0.85	***0.65***
RecC3	0.85	0.86	0.77	0.80	***0.65***
RecC4	0.80	0.89	0.83	0.80	***0.60***
RecC5	0.85	0.86	0.78	0.86	***0.65***
RecC6	0.80	0.84	0.71	0.78	***0.55***
RecC7	***0.64***	0.78	0.80	0.69	0.83

(Fig. 5-3) or represented by D_{ps} (Table 5-3). Three putative groups of recombinants are apparent in both analyses. Recombinant group A shows greater similarity to Virasoro than the typical clone, recombinant group B shows a clear contribution from *P. dilatatum*

ssp. *flavescens*, and recombinant group C is more heterogeneous and appears to share more alleles with Virasoro than the typical clone. The multilocus genotypes shown in Fig. 5-4 are in complete agreement with this. In agreement with the estimated allele admixture proportions shown in Fig. 5-3, the genotypes in groups A and B can be almost fully accounted for by a cross between typical clones A or P and the individuals of Virasoro and *P. dilatatum* ssp. *flavescens* included in Fig. 5-4.

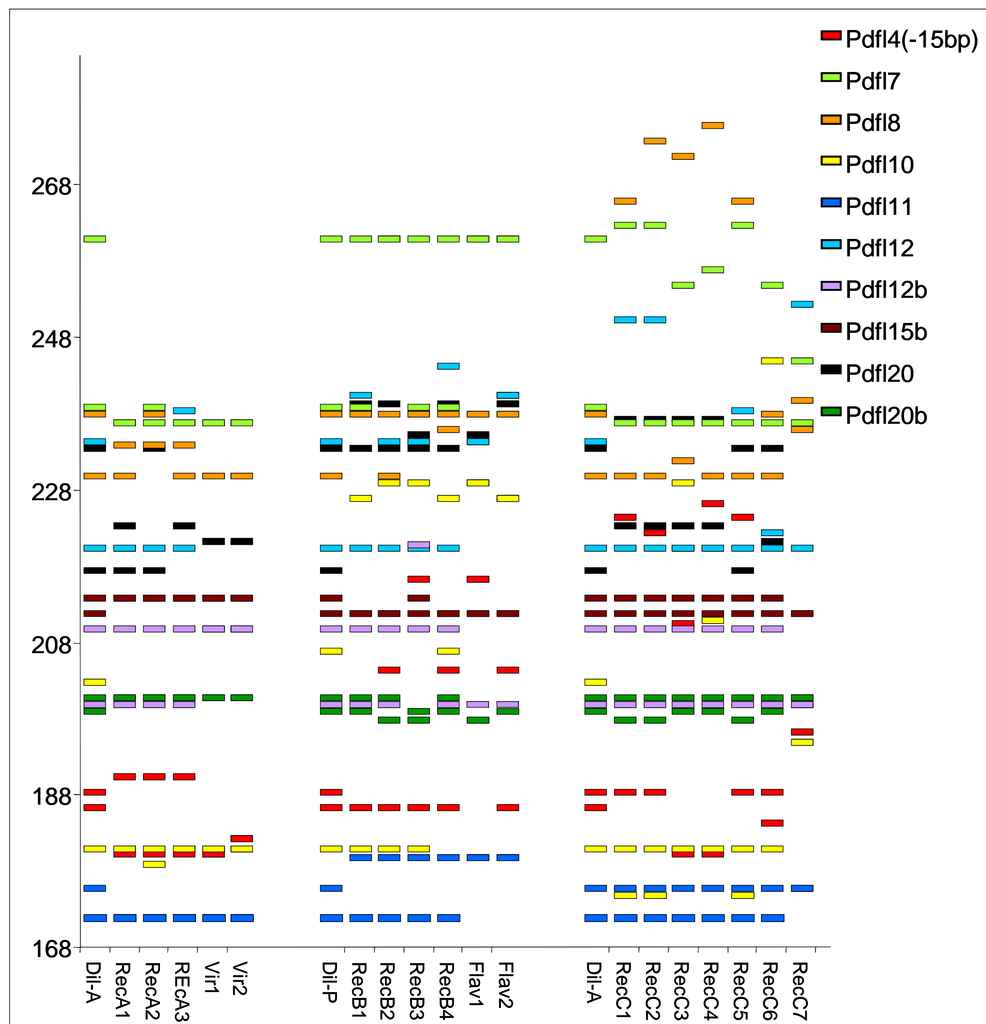

Fig. 5-4. Multilocus microsatellite genotypes for the recombinant genotypes of pentaploid *P. dilatatum*. The nearest genotype from the typical clone (*A* or P) is shown next to each recombinant group. Two individuals of *P. dilatatum* ssp. *flavescens* and Virasoro are shown next to each of the clusters they appear to be related to for comparison. The highly variable locus Pdfl15 is not shown.

Furthermore, recombinants of group B are found in the area of co-occurrence of pentaploids and *P. dilatatum* ssp. *flavescens*, and the recombinants of group A are found either in the area of distribution of Virasoro or near it (Fig. 5-5).

The putative recombinants of group C present a different situation. First, a clear contribution of an existing tetraploid to their genotypes was not found. Second, their geographical distribution does not overlap with that of any currently known tetraploid, except for genotype C5, and third, they show allele sizes that are beyond the range so far detected in the tetraploids analyzed (longer allele of Pdfl15 in C1, 2 and 7; longer allele of Pdfl8 in C1-5; shorter allele of Pdfl10 in C1, 2 and 5) (Table 5-2). Genotype C5, in spite of its geographical location, does not show a clear contribution of Vacaria (see Fig. 5-4 and Table 5-2).

Discussion

The "Typical" Clone

One of the difficulties encountered in this data set was the discrimination of the extent of the variability caused by mutation from that due to hybridization. Although it was evident from the data that a single common genotype comprises the majority of the accessions analyzed, hybridization to a homozygous source could be confounded with a high degree of mutagenic activity. The genotypic matrix contained 22 different alleles because the common genotype is heterozygous at all the loci assigned to the I or J genomes. If this clone crossed to any unrelated genotype, a maximum of 11 allele-size differences would be expected. However, the two tetraploids with which hybridization was hypothesized (Virasoro and *P. dilatatum* ssp. *flavescens*) share a number of alleles with pentaploid *P. dilatatum*; hybridization to either of them would then produce fewer than the maximum of 11 differences. With a low number of hybrids as detected here,

sexual events would not necessarily cause conflict in the data matrix and could maintain a hierarchical structure in spite of recombination. More systematic character compatibility approaches to detecting recombination in apomicts rely on the generation of this conflict (Mes 1998) and only provide an approximation to the quantification of sexual reproduction (Mes et al. 2002). In spite of the above, our case by case analysis has probably resulted in a very realistic distinction of clonal variants and recombinants.

In well-studied apomictic plant systems like *Taraxacum*, apomicts tend to behave as good overseas colonizers (van Dijk 2003); however, unlike *P. dilatatum*, native ranges usually contain an assemblage of independently generated clones regardless of how widespread they may be (van der Hulst et al. 2000). Regardless of the detection of a certain number of recombinants, most of pentaploid *P. dilatatum* comprises a single clone with its somatic variants; moreover, most of the individuals collected outside the native range of the species have shown the same genotype when analyzed for this set of loci. The actual extension of this clone around the globe is not known but it has been shown here to be present on every continent. The current collection is not suitable for estimating the proportion of typical genotypes within the native range because most of the samples were collected as individual off-types or as different types within a morphologically variable population. It is likely then, that recombinants are less frequent than detected here. It is remarkable that the typical clone was present in all populational samples containing recombinants except population 10. This population, however, was collected and annotated as *P. pauciciliatum*, so it is likely that if typical individuals were present at the site, they may have been avoided.

It follows from the above that the dominance of this clone is not currently being challenged, not even at the local scale. Whether this clone is a one-of-a-kind highly successful interspecific combination or the result of selection among numerous related clonal lineages (Parker et al. 1977), typical pentaploids may represent a General Purpose Genotype (GPG) (Lynch 1984). The GPG hypothesis is even more appealing for a long-lived perennial grass with low seed production than for the insect populations with rapid turn-over for which it was advanced. On the other hand, clones are not necessarily evolutionarily inactive (Loxdale and Lushai 2003) or devoid of marker diversity (Mes et al. 2002). The mutational diversification of the typical clone of *P. dilatatum* detected with microsatellites is significant, and it is likely that the biotype contains a great unexplored wealth of genetic variability.

Distribution of Clonal Diversity in the Native Range

It is well established that the plant communities of the Uruguayan grasslands have been dramatically altered by the introduction of domestic cattle, with current conditions favoring semiprostrate warm-season grasses (Rodríguez 2003). Among the biotypes of *P. dilatatum*, it is particularly the pentaploids that match such ecological requirements. It has long been thought (J.C. Millot pers. comm. 1990) that pentaploid *P. dilatatum* originally had a much more restricted distribution and has rapidly spread since the introduction of domestic cattle in the 17th century. Ravines and waterways in the hilly landscape of eastern Uruguay currently provide refugium conditions for subtropical flora (Grela 2003). Independent lines of ongoing research (Speranza and Solís-Neffa, Vaio and Speranza unpub. res.) indicate that the herbaceous components in this region are also genetically differentiated from surroundings populations, suggesting that the area may have acted as a refugium during drier and cooler climatic phases for herbaceous species

that are currently more widespread. In this scenario, the typical clone may have originated and spent most of its history in the suggested (or any other) restricted geographical range, and generated most of its somatic variants before spreading during the past three centuries. Such rapid expansion can account for the lack of geographical structure in the distribution of genotypes and the multiplicity of clonal variants in eastern Uruguay.

Hybridization and its Genetic Consequences

In areas of sympatry with compatible sexual species, the typical clone seems to be developing a reservoir of alternative forms which may play an important role in securing the survival of the biotype if the climatic or ecological conditions in the area changed. This pattern has been reported for several agamic complexes (Hörandl 2004) and recently for *P. notatum* (Daurelio et al. 2004). The current contact between pentaploids and some tetraploids, on the other hand, may be secondary and relatively recent, as reflected by the geographical location of hybrids with Virasoro and ssp. *flavescens*. The presence of hybrids in these areas represents indeed the ongoing transfer of genetic variability from a locally adapted selfer like *P. dilatatum* ssp. *flavesencens* to a genetically aggressive advancing species, much in the fashion of the compilospecies concept proposed by Harlan (1963). The facts that Virasoro and ssp. *flavescens* are selfers, highly homozygous (Chapter 3, Chapter 4, Hickenbick et al. 1992) and consequently probably carry a low genetic load has further implications in the same direction: the incorporation of an IJ gamete from either of them is equivalent to the incorporation of the whole genotype of a locally adapted counterpart and a significant simultaneous reduction of the genetic load of the resulting apomict.

The Mechanism

The formation of euploid apomictic pentaploids by hybridization between tetraploids and pentaploids has been achieved experimentally with a relatively high frequency (Speranza 1994 unpub. res.). Bennett et al. (1969) reported a very low crossability for the tetraploid x pentaploid cross (0.04%) but we obtained almost 1% crossability (4 hybrids out of 455 emasculations), nearly ten times as high as the average of our crosses to hexaploids (Chirú) over three seasons (17 hybrids out of 11,100 emasculations). Of the 4 hybrids we obtained, 2 were pentaploid and apomictic and were almost indistinguishable from their pentaploid parent, one was aneuploid (2n=45) and sexual, and the fourth one was heptaploid (2n=70). When analyzed with the same markers used in this study, the genotypes of the pentaploids were as expected for an F_1 hybrid. Euploid hybrids can be generated if pentaploids can produce normally reduced gametes for their I and J genomes that carry a full complement of X chromosomes. When the meiotic fate of the X chromosomes was observed in the pentaploid pollen donor, they were seen to lag during anaphase I, stay condensed and end up in one of the dyad members at the end of telophase I. Most lagging chromosomes were observed to form a single micronucleus with a much higher frequency than expected by chance. Unexpectedly, this chromatin cluster organized itself into a secondary metaphasic plate, and sister chromatids segregated synchronically with the main group of chromosomes (Mazzella and Speranza 1997, M. Klastornick, unpub. res.)

Hybrids receiving less than the 10 X chromosomes in this kind of cross apparently do not inherit apomixis. The 45-chromosome plant we recovered from a cross between the tetraploid *P. dilatatum* ssp. *flavescens* and pentaploid *P. dilatatum* was weak and sexual and the F_3 individuals reverted to a phenotype very similar to that of *P. dilatatum*

ssp. *flavescens*. Bennett et al. (1969) and Burson (1995) also reported plants with incomplete X genomes that reproduced sexually, indeed it was this last hybrid that was later used to assess the homology between the X genomes of pentaploid *P. dilatatum* and Chirú (Burson 1991). It can be safely concluded that the apomixis genes are located on the X chromosomes but, are all ten X chromosomes necessary for apomixis? This cannot be answered for sure yet, but most of the artificial and natural hybrids reported have a full complement of X chromosomes. Once this type of euploid IJX gamete can be produced, their frequency need not be too high to explain the formation of apomictic pentaploids by tetraploid x pentaploid crosses. Events in which apomixis has not been successfully passed on will not be observed in nature, and the establishment of apomixis seems to require at least most of the chromosomes of the X genome.

How Many Times?

The origin of the typical pentaploids has not been established yet; however, the completely heterozygous genotype of the pentaploids suggests at least one round of hybridization after apomixis was established (Chapter 4). The microsatellite genotype of *P. pauciciliatum* is congruent with a pentaploid x diploid cross, and evidence for the derivation of Uuruguaiana, Chirú, and a heptaploid hybrid from the pentaploid form is even more compelling. Here, at least four independent events of crosses to ssp. *flavescens*, three to Virasoro and an undetermined number to at least one more unidentified tetraploid are well supported by the data. Furthermore, the hybrids reported by Machado et al. (2005) may well fit the same pattern. In all these crosses, the same set of ten chromosomes of the X genome has been transmitted without changes or recombination. Our results do not show any evidence that the process will come to an end in the near future; the X genome needs only maintain its ability to code for apomixis

to secure its survival regardless of how many times it may be transferred onto different IIJJ backgrounds . Pentaploid *P. dilatatum*, far from being the fortunate single product of a random encounter between a dwindling hexaploid and a geographically restricted tetraploid seems to be an aggressive compilospecies with an unpredictable evolutionary future.

CHAPTER 6
CONCLUDING REMARKS

The work presented in this dissertation has provided answers to several questions about the evolutionary mechanisms operating within the Dilatata group of *Paspalum*.

The apomictic complex within the group has been shown to be evolutionarily active and still generating new variants. The focus has shifted from the hexaploids, which were thought to have played a central role in the formation of new apomicts, to the pentaploids, whose evolutionary potential has been clearly demonstrated. At the same time, a mechanism was proposed by means of which an imbalanced polyploid can be involved in several sexual events and reproduce itself while enhancing its evolutionary potential in the process. This phenomenon, even though it does not seem to have been too frequent, is reminiscent of the compilospecies model, in which a genetically aggressive entity is able to engulf the local adaptation generated by related species and advance over their areas of distribution. The behavior of the X genome of the Dilatata group is somewhat unprecedented. Even if some of the transmission events that were hypothesized in Chapters 4 and 5 could be given an alternative explanation, it seems that the same set of ten chromosomes has been transmitted unchanged and unrecombined several times since the origin of the group. This mechanism is a powerful explanatory tool which can account for the multiplicity of forms currently known in the group and predicts that several more will be found.

Before this work, the apomicts in the Dilatata group comprised one tetraploid, one pentaploid and two hexaploids, each one thought to be a unique event. Only recently had

the possibility that the group contained more forms been suggested by Machado et al. (2005). My analysis shows that the group contains multiple hybrids, all of which are similarly divergent and deserve the same rank as the four biotypes that had been identified based on chromosome numbers. Each one of the four previously known hybrids had been treated by applied researchers as a different entity and analyzed accordingly. It can easily be predicted that within the next few years the number of new “biotypes” will have increased at least by one order of magnitude, and the task of evaluating them may have also increased accordingly.

The collection and management of the accessions should also accompany the new perspective. The recombinant individuals have most often been found intermixed with representatives of the typical pentaploid clone. A collection of highly divergent apomicts is not a valid management unit in a clonal breeding program. To be able to manage or use these new “biotypes,” individual progeny accessions should be stored and characterized. This strategy proved successful in reflecting the variability in the populations that had been identified as variable by J.C. Millot as was shown here. The current collections should be restructured and the component clones of each accession identified and separated if they are ever to be used for the purpose they were intended.

No attempt to make taxonomic decisions was made in any of the parts of this dissertation. However, the issue seems unavoidable. The relationships among the tetraploid components of the group have not been clarified; however, the fact that they share an identical sequence for all the chloroplast regions that were analyzed (except for the addition or deletion of a few adenines in a poly A-tract) clearly stresses their relatedness.

Two of them, *P. dasypleurum* and *P. urvillei*, have been acknowledged as different species. The remaining three entities, currently considered biotypes (or subspecies) of *P. dilatatum* are genetically distinct, and no gene flow has been recorded except in artificial crosses. A comprehensive morphological study including an exhaustive determination of their distribution ranges is still lacking, but it is likely that no biological connections other than their relatedness will be found among them. Perhaps, from the point of view of convenience, they should first become named subspecies as was suggested in Chapter 4. The apomicts, on the other hand, may all be connected by a few sexual events, and it is possible that their ancestries trace back to a single population or even a single individual, and they can be considered to be the product of several attempts by the same entity to secure its evolutionary future. Even if the informal biotype designations in use are still appropriate for the hexaploids, the expression "common" to designate the pentaploids has lost all meaning. The predicted multiplicity of hybrids will make the naming of all of them impractical and meaningless.

The origin of the Dilatata group as a whole remains as obscure as it was. In fact, the arrangement of the species with known genomic formulae in the preliminary phylogeny that was shown in the Chapter 1 suggests that we may be a long way from identifying the direct donors of the two basic genomes on which the complex is constructed. The complex relationships among the members of the Quadrifaria group that were outlined in Vaio et al. (2005) cautions about the current interpretation of cytogenetic affinities. In spite of this, the first necessary step has been taken. The construction of a phylogeny of the genus is under way.

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BIOGRAPHICAL SKETCH

Pablo Speranza was born in Montevideo, Uruguay, in 1967. Since his very early childhood he showed great interest in biology. Unsure of what the future may hold he registered at the University of the Republic in Uruguay both to receive a license in biology and a degree in agronomy. In 1989 while taking a required course in plant breeding he met Prof. J.C. Millot who was very influential in his career and later served as the chair of his Agronomy thesis. In 1990, after having passed the exam to the Plant Breeding course he started working as a teaching assistant for the course. He received his degree of Ingeniero Agrónomo in 1995 after completing a thesis on the cytogenetics and agronomic characterization of an interspecific *Paspalum* hybrid produced by Prof. Millot. He has been working in the Department of Plant Biology at the Facultad de Agronomía in Montevideo, Uruguay, since then under the supervision of C. Mazzella who later served as the chair of his master's dissertation committee. His Master's dissertation included research in molecular cytogenetics in *Paspalum dilatatum* and related species which was carried out at the John Innes Research Center in Norwich, England, under the supervision of Prof. J.S. (Pat) Heslop-Harrison. Pablo obtained his master's degree in biology in 1999 from the Programa de Desarrollo en Ciencias Básicas of the University of the Republic.

In 1991 Pablo obtained a faculty position at the Department of Plant Biology and taught plant breeding until 1999. In order to pursue his academic career he decided to obtain a PhD degree abroad. In 2000 he was awarded a Fulbright scholarship and started a PhD program at Washington State University under the supervision of Pamela Soltis. In

2001 Pamela and Douglas Soltis moved to the University of Florida and Pablo completed his PhD program here in August 2005. In August 2005, he will be moving back to Uruguay and will resume his academic career at the University of the Republic, where he already has several collaborative projects under way.