Use of Genuine Sources of Ergot Resistance in Species of the Dilatata Group of Paspalum

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Abstract
Paspalum dilatatum Poir. (dallisgrass) is an excellent C4 forage for summer production, highly productive with a wide distribution within warm-temperate regions. *P. dilatatum* is native to South America with special relevance for milk and meat production since its forage quality is superior to that of other C4 forage grasses and it shows tolerance to frost and water stress. In situations of temperate and warm temperate climate, the presence of these species plays a key role in the ecological stability of the system due to the complementarity of the growth cycles between winter and summer grasses. *Claviceps paspali* (ergot) is a fungus that mainly parasitizes *Paspalum spp.*, generating a structure called sclerotia, in which indole-diterpenoid alkaloids are isolated. Despite the excellent forage characteristics of *Paspalum* species, there is a need for the generation of varieties able to overcome two major limitations that were identified early on in this species, such as ergot susceptibility and seed production. With this objective, selected genotypes were crossed between apomictic and sexual species of different ploidy using *P. malacophyllum* as a source of immunity. Immunity to ergot has been evaluated in the field for two years in different representative regions of Uruguay and the accessions that did not get sick were selected as pollen donors. Crosses made with *P. flavescens* showed a germination percentage ranging between 1.7 and 7.09, while in *P. dilatatum* var. Chirú the range was reduced between 0.99 and 1.25 according to the employed parents. The hybrid nature of the progeny is being verified by microsatellites and functional markers associated with immunity and DNA content estimated by flow cytometry. This work aims to generate the basis to transfer immunity from *P. malacophyllum* in selected genotypes of *Paspalum* species predominant in Pampa biome and to improve seed production.

Introduction
Paspalum species of the Dilatata group are native to the Pampa Biome region. In Uruguay the are several well characterized species from this group, including the common pentaploid apomictic biotype *P. dilatatum* var. *dilatatum*, the hexaploid biotype 'Chirú' (*P. dilatatum* var. Chirú), which has been early selected for its outstanding agronomic characteristics, and the sexual tetraploid *P. flavescens*. Commercial development of *P. dilatatum* varieties has been halted by their high ergot susceptibility, which greatly reduces seed yield and quality, and where no genuine sources of resistance are found within the species. However, early studies have indicated the presence of ergot immunity in *P. malacophyllum* (Bennett and Bashaw, 1960), a tetraploid apomictic species with low grazing adaptation but opening the possibility for interspecific breeding. With the aim of improving ergot resistance and seed production through interspecific hybridization we pose three specific objectives: i) test the ergot immunity of *P. malacophyllum* to local strains of *Claviceps paspali* to find a genuine source of resistance/immunity to ergot; ii) create *Paspalum* interspecific hybrids by crossing Chirú and *P. flavescens* with *P. malacophyllum* accessions as pollinators; iii) develop molecular markers to recognize the hybrid nature of the progeny.

Methods
Ergot susceptibility screening

Greenhouse and field trials were conducted in *P. dilatatum* var Chirú, *P. flavescens* and *P. malacophyllum* (PI 508796, PI 508797 PI 508800) to evaluate ergot incidence and severity. Field trials were placed in 5 locations of Uruguay and during March and April 2021, plants were visually inspected for ergot infection. Greenhouse infection-trails were conducted in INIA Las Brujas in the same dates. The incidence and severity of ergot symptoms were determined based on: I) percentage of florets with honey or sclerotia over the total number of florets per plant. II) Evaluation of production of honeydew.

Interspecific crosses

Apomictic hexaploid pistillate plants of *P. dilatatum* var. Chirú and sexual tetraploids of *P. flavescens* were used as pollen receptor, while three immune accessions of *P. malacophyllum* were used as fresh pollen donors. Emasculation was performed early in the morning in the presence of natural light and high humidity conditions were set in the greenhouse of INIA Las Brujas from March to October 2022.

Molecular markers development

Fourteen functional markers associated with immunity and identified in the draft genome of *P. malacophyllum* and 15 microsatellites (SSR) designed for *P. flavescens* were employed (Speranza and Malosetti, 2007).

The PCR reaction mix optimized for both sets of markers was carried out in a volume of 25 µL that contained: 100 ng of DNA, 1× MyFi Mix (Meridian Bioscience, Catalog Number BIO-25049) and 0.4 µM of each primer. In addition to the forward and the reverse primers, the PCR reaction mix for the SSRs set also included a universal fluorescent labelled M13(-21) primer (Schuelke, 2000).

Flow cytometry

DNA content in the progeny of the interspecific crosses was estimated by flow cytometry. Nuclear suspensions were prepared according to Galbraith et al. (1983) using woody plant buffer (WPB). Nuclei were stained with 25 µL propidium iodide (Sigma, P1304MP), plant cells were immediately analyzed. DNA content analysis was performed on a Sysmex- Partec CyFlow® Space flow cytometer (Faculty of Agronomy, Uruguay) with an Innova 300 laser at 488 nm and CELLQuest software. At least four DNA estimations were performed for each plant (3000 to 5000 cells per analysis). Nuclear DNA content (2C) was calculated as: (Mean of sample peak/mean of standard peak) x 2C DNA content of standard (pg). The 2C DNA content data were analyzed with the FloMax statistical package.

Results and Discussion

Ergot susceptibility screening

Greenhouse analyses performed with highly virulent isolates, and open field trials, corroborated that *P. dilatatum* var Chirú and *P. flavescens* are very susceptible to ergot under our environmental conditions, while the three *P. malacophyllum* accessions tested were immune (data not shown). This confirms that *P. malacophyllum* could be a valuable genetic source for Ergot resistance as stated by Bennet and Bashaw (1960).

Interspecific hybridization

Although many efforts have been made to improve ergot resistance using interspecific crosses of *Paspalum*, none of these reached commercial phase. Among the difficulties for performing hybridization, apomictic species, different ploidy levels, and synchronization of flowering, are the main ones. Besides that, this technique requires lot of manual dedication, patience and it is time consumption. However, the benefits can justify the effort if we consider those mentioned by Bennett and Bashaw (1960) such as transferring immunity, re-establishing sexuality, and observing heterosis. Table 1 and 2 summarizes the number of crosses made in *P. dilatatum* var. Chirú x *P. malacophyllum* and *P. flavescens* x *P. malacophyllum*, the hybrids obtained, and a preliminary characterization based on DNA content, similarity plant phenotype observed and marker profile observations.

Table 1. Crossings carried out from February to October 2022 at INIA Las Brujas. The presence of hybrids was determined with specific functional of *P. malacophyllum* and transferable microsatellites.

Crossing	Vain/Empty	Full	Total	Fertility rate (%)	Sown seeds	Germination rate (%)	Hybrids (quantity)
Chirú x PI 508796	399	23	422	5,45	412	1,21	0
Chirú x PI 508797	1461	157	1618	9,70	1600	1,38	2
Chirú x PI 508800	749	25	774	3,22	426	0,94	0
Flavescens x PI 508796	384	190	574	33,10	530	1,70	w/i
Flavescens x PI 508797	1147	241	1388	17,36	1136	4,67	w/i
Flavescens x PI 508800	686	352	1038	33,91	706	13,31	w/i

w/i: without information

Table 2. Preliminary characterization of DNA content, phenotype inferred by visual appearance, and microsatellites profiles.

Crossing	DNA content (pg)			Phenotype	Microsatellites			
	2C value	Estimated Ploidy level	Inferred Genomic Formula		Pf7	Pf10	Pf15	Pf 28
Chirú	3,72	6X	II JJ XX	Ch	Ch=Ml	Ch	Ch	Ch
PI 508797	3,93	4X	MM MM	Ml	Ch=Ml	Ml	Ml	Ml
Cr 1	3,65	5X	I J X MM	Ml	I*	I	Ml	I
Cr 2	3,74	5X	I J X MM	Ml	I*	Ml	Ml	I
Cr 9	3,04	Possible Chirú auto	II JJ XX	Ch	I*	I	I*	I*
Cr 10	3,03	Possible Chirú auto	II JJ XX	Ch	I*	I	I*	I*
Cr 11	5,44	8X (5,68)	II JJ XX MM	Ch	Ch=Ml	Ml	I	Ml

I: individual, **I***: individual equals to each other, **Ch**: *P. dilatatum* var. Estanzuela Chirú, **MI**: *P. malacophyllum*

Seed quality is usually evaluated by considering several components. One of the main ones is physical quality, understood as the proportion of germinable or viable seeds. As in other panicle species, it is common to find a high proportion of empty seeds. In Chirú, the presence of empty seeds may be associated with apomictic reproduction, and it is known that sexual biotypes such as *P. flavescens* usually have a higher proportion of full seeds than apomictic biotypes. A preliminary observation seems to indicate that there are differences between pollen donor plants for each pistillate species. Furthermore, it would be easier to make crosses with sexual species than with apomictic species, as can be seen from the fertility percentage obtained. But we must also consider the endosperm balance number (EBN) as stated by Johnston and Hanneman (1980) when obtaining an interspecific hybrid. Despite the higher observed fertility with *P. flavescens*, no hybrids were detected so far, suggesting that embryo rescue might be needed.

This percentage for Chirú is like that observed for the expression of sexuality in the order of 5%. In the cross *flavescens* x *malacophyllum*, although the fertility rate is similar for PI 508796 and PI 508800, the germination percentage of PI 508796 is much lower (Table 1), compromising future progenies of this cross.

Table 3. Information on microsatellites used in this work.

Marker Name	Primer Sequences (5'-3')	Repeat Unit	Tm (ºC)	GC (%)	Expected Size (bp)	Observed Size (bp)
Pdfl-10	Fw:GCTCATCAAATATGACTGAACCA	$(TG)_8CG(TG)_{21}$	69.3	44	142	Ch: 181
	Rv:TCTTACGTCCCACCCAAATC		60.3	50		M: 272
Pdfl-15a	Fw:AACCACTGTGTGAAGCTTGCTA	$(GT)_2GC(GT)_{43}$	70.4	48	152	Ch: 214
	Rv:TGTGCACACTCATCGAAAGA		58.2	45		M: 161
Pdfl-28a	Fw:AAAATACCCGTGCGTTGCTA	$(TG)_{32}$	69.6	47	159	Ch: 159
	Rv:CCACGCCATGTCGTCTACTA		60.9	55		M: 173

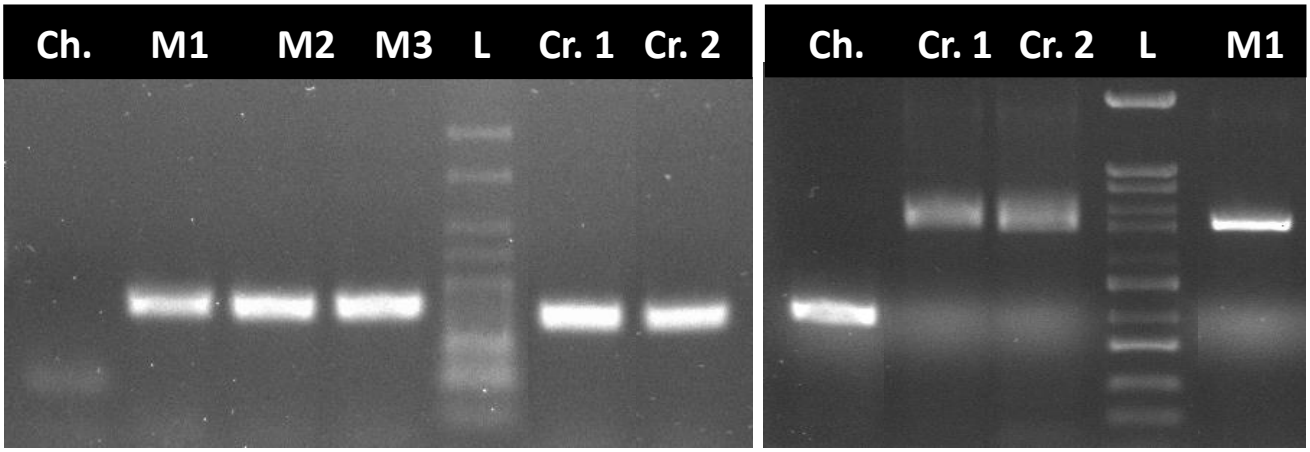
Ch: *P. dilatatum* var. Estanzuela Chirú, **M**: *P. malacophyllum*

Molecular microsatellites and DNA content determination

Microsatellite transferable markers (Table 3) proved to be good tools to recognize the hybrid nature of the progeny. Considering the putative hybrids obtained (Table 2 and Figure 1), the microsatellites allowed us to recognize the presence of paternal alleles in the progeny. Considering that it is a cross between polyploids (Chiru 2n=6x and *P. malacophyllum* 2n=4x), the DNA content values would allow us to infer the ploidy level of the hybrids compared to the known genomic constitutions. According to the expected fertilization of reduced

embryosacs and not reduced ones, as established in Table 1, a possible genomic formula was established that needs to be corroborated.

Figure 1. Agarose 3% gel electrophoresis showing amplification profiles obtained with microsatellite marker 15a and functional 012927.



Left: Microsatellite 15 a. Right: Functional marker012927

Ch *P. dilatatum* var. Estanzuela Chirú. **M1** *P. malacophyllum* 508797. **M2***: P. malacophyllum* 508796. **M3**: *P. malacophyllum* 508800. **Cr.1**: Chirú x 508797. **Cr2:** Chirú x 508797. **Ladder Left**: HyperLadder 25bp (Meridian Bioscience); **Ladder Right** 100bp (Maestrogen).

Conclusions and/or Implications

The hybrid nature of the progeny was confirmed by using molecular markers and flow cytometry. Obtaining hybrids in the cross between *P. dilatatum* var. Chirú and *P. malacophyllum* opens the door for further studies on ergot response and the mode of reproduction of the progeny.

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