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Origins of polyploidy in *Paspalum stellatum* and related species (Poaceae, Panicoideae, Paspaleae) inferred from phylogenetic and cytogenetic analyses

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Paspalum stellatum is widely distributed in the American continent and it is known to include diploid cytotypes ($2n = 2x = 20$) and polyploid cytotypes ($2n = 32$ and 52). It is closely related to *P. eucomum*, *P. malmeanum* and *P. schesslii*. After chromosome counting and geographical mapping of the different cytotypes, phylogenetic relationships between them were explored in order to infer the origin and evolution of the polyploid complex *P. stellatum* and related species. Additionally, several unusual chromosome numbers are here reported for *P. stellatum* ($2n = 30$, 46, 48, 56 and 60) from the Brazilian cerrado. Phylogenetic analysis using plastid DNA showed that the cytotypes of *P. stellatum* split into two clades, one of which included all accessions with $2n = 32$ and *P. schesslii*. Using ITS for phylogenetic analysis showed that *P. schesslii*, *P. malmeanum* and *P. eucomum* were grouped together as expected. The origin of the cytotype $2n = 32$ of *P. stellatum* may have involved *P. schesslii* as one of the putative progenitors with a diploid cytotype of *P. stellatum* as the other.

ADDITIONAL KEYWORDS: chromosome count – diploid hybrid speciation – ITS – molecular phylogeny – plastid DNA – polyploid hybrid speciation.

INTRODUCTION

Polyploidization is a common event in plants and has had a major role in species diversification during angiosperm evolution (Estep *et al.*, 2014; Murat *et al.*, 2017). Since polyploidization brings reproductive isolation from diploid progenitors, it represents a barrier to gene flow (Husband & Sabara, 2004). Polyploidy is also associated with the origin of major angiosperm linages; Jiao *et al.* (2011) proposed that at least two events of polyploidization may have occurred in the early-diverging monocots, leading to the diversification of the grasses. In Poaceae, many genera also combine asexual and sexual reproduction with polyploidy in the same species or group of species to give rise to polyploid complexes. These include sexual and apomictic counterparts with different ploidies in which, in most cases, diploids are sexual and polyploids are apomictic (Hörandl *et al.*, 2008; Sartor *et al.*, 2013; Hojsgaard *et al.*, 2014).

The species-rich genus *Paspalum* L. (Poaceae) belongs to tribe Paspaleae, characterized by having a chromosome base number $x = 10$. With *c*. 350 species, it presents an outstanding combination of complex reproductive systems and a wide range of

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ploidies, the most common case being the occurrence of sexual diploids ($2n = 2x = 20$) and conspecific apomictic tetraploids ($2n = 4x = 40$) (Burson & Quarin, 1992; Norrmann *et al.*, 1994; Hojsgaard *et al.*, 2009; Rua *et al.*, 2010).

Several of the polyploids have a hybrid origin and nearly 80% of the species in the genus have at least one polyploid cytotype (Quarin, 1992; Sartor *et al.*, 2013). Although considerable research efforts have been devoted to *Paspalum*, relatively few species have been studied at a detailed level of analysis and the possibility that a large proportion of polyploid members could potentially have been involved in the formation of large hybrid complexes should not be ruled out. Moreover, molecular phylogenetic hypotheses are still poorly resolved (Rua *et al.*, 2010; Scataglini *et al.*, 2013) and many questions about phylogenetic relationships in the genus remain unanswered.

Paspalum stellatum Humb. & Bonpl. ex Flüggé, as currently recognized, is widely distributed in fields and savannas on sandy or rocky soils from Mexico to southern Brazil, Paraguay, north-eastern Argentina and Uruguay (Denham *et al.*, 2002). The taxon is known to include cytotypes with $2n = 20$, 32 and 52 (Honfi *et al.*, 1990; Killeen, 1990; Sader *et al.*, 2008; Bonasora *et al.*, 2015), although relatively little information on chromosome counts is available and it covers only a minor portion of the known geographical range of this species.

Often, several currently recognized taxa of *Paspalum*, both apomictic and sexual, are involved in the formation of a single polyploid agamic complex (Vaio *et al.*, 2005; Speranza, 2009). For this reason, species that are genetically and morphologically related to polyploid apomicts have been included in the analysis of such complexes.

Paspalum stellatum, P. eucomum Nees ex Trin., *P. malmeanum* Ekman and *P. schesslii* Bonasora & G.H.Rua constitute a group of closely related species because they share conjugate racemes with variable winged rachises and densely pilose spikelets (Denham *et al.*, 2002). In this group, *P. schesslii* is known to have $2n = 2x = 12$ chromosomes (Bonasora *et al.*, 2015). Such a chromosome number is unusual in *Paspalum*, in which, with few exceptions, the chromosome base number is $x = 10$. Prior to the recent discovery of *P. schesslii* (Bonasora *et al.*, 2015), the only other reported species with $x = 6$ was *P. almum* Chase (Quarin, 1974); therefore a relationship between the latter species and the $2n = 32$ cytotype of *P. stellatum* has been proposed (Sader *et al.*, 2008; Bonasora *et al.*, 2015).

Hybridization events are not easily described in tractable mathematical models of speciation (Meimberg *et al.*, 2009; Estep *et al.*, 2014). The evolutionary history of polyploid complexes comprises one or more reticulation events, making the adequate reconstruction of a hierarchical phylogenetic tree difficult (Linder & Rieseberg, 2004). Studies based on detecting inconsistencies between plastid DNA and ITS sequences have proved helpful as a first approach to clarify the complicated reticulate evolutionary patterns of hybrid complexes driven by polyploidy and apomixis (e.g. Soltis *et al.*, 2008; Russell *et al.*, 2010; Majeský *et al.*, 2012; Krak *et al.*, 2013; Róis *et al.*, 2016).

The aims of the present research are to shed some light on the pathways that gave rise to the polyploid cytotypes of *P. stellatum* and to identify their putative progenitors and the contact zones by combining geographical and cytogenetic information with phylogenetic analysis based on nuclear and plastid markers.

MATERIAL AND METHODS

Accessions of *P. stellatum* and related species were collected from natural populations in Argentina, Paraguay, Bolivia and Brazil in field trips during 2011 and 2012, covering the known distribution in southern South America. Information on chromosome numbers was obtained from the cytological indices Index of Plant Chromosome Numbers (IPCN) and the International Organization of Plant Biosystematics (IOPB) chromosome number reports. Also, our new counts were taken into account (Table 1). Localities were georeferenced using DivaGis 7.5.0.0 (Hijmans *et al.*, 2005) (Table 1, Fig. 1).

Chromosome counts

For mitotic chromosome analysis, root tip meristems were obtained from plants cultivated in pots. The meristems were pre-treated with a saturated solution of α-bromonaphthalene for 2.5 h at room temperature. Fixation was performed in 3/1 (v/v) ethanol/acetic acid for at least 24–48 h. After hydrolysis in 1M HCl for 10 min at 60 °C and staining in Schiff´s reagent following the standard method (Feulgen & Rossenbeck, 1924), the root tips were squashed in a drop of acetic carmine. After freezing with dry ice, cover slips were removed and preparations were mounted in Euparal.

Chromosome plates were observed under a Leica Axioplan microscope and analysed using a camera and image analysis software. The chromosome number was determined from at least ten cells per root tip per accession.

DNA extraction, amplification and sequencing

DNA was isolated from fresh leaves or silica-gel-dried leaves following the modified CTAB protocol of Doyle & Doyle (1987), adapted for small amounts of plant material. The nuclear ribosomal ITS region and four

Table 1. Species, accessions, localities, altitudes, longitudes, latitudes and chromosome counts for *Paspalum stellatum* and related species. The table includes new chromosomes numbers reported here and previous counts reported in the literature

Species	Voucher	Country	Locality	Altitude (m)	Latitude	Longitude	2*n*	Reference for chromosome count
Paspalum eucomum	*Rua, G.H. et al. 939*	Brazil	GO – Mineiros	872	–17,379	–52,924	30	Bonasora *et al.* 2015
Paspalum eucomum	*Rua, G.H. et al. 1124*	Brazil	PR – Ponta Grossa	934	–25,028	–50,077	32	Bonasora *et al.* 2015
Paspalum malmeanum	*Killeen, T. J. 2478*	Bolivia	SCZ – Ñuflo de Chávez	480	–16,2	–62	20	Killeen 1990
Paspalum malmeanum	*Morrone, O. et al. 4991b*	Bolivia	SCZ – Ñuflo de Chávez	480	–16,209	–62	20	Sede *et al.* 2010
Paspalum malmeanum	*Rua, G.H. et al. 1040*	Bolivia	SCZ – Ñuflo de Chávez	483	–16,185	–62,015	20	Bonasora *et al.* 2015
Paspalum schesslii	*Rua, G.H. et al. 991*	Brazil	MG – Cuiabá	192	–15,464	–56,051	12	Bonasora *et al.* 2015
Paspalum schesslii	*Rua, G.H. et al. 975*	Brazil	MT – Porto Estrela	212	–15,666	–57,273	12	Bonasora *et al.* 2015
Paspalum stellatum	*Quarín, C.L. et al. 3714*	Argentina	CNT – Capital		–28,502	–56,055	32	Honfi *et al.* 1990
Paspalum stellatum	*Quarín, C.L. et al. 3852*	Argentina	ERS – Federación		–30,706	–58,158	32	Honfi *et al.* 1990
Paspalum stellatum	*Honfi, A.I. et al. 1168*	Argentina	MSN – San Ignacio	114	–27,323	–55,535	32	Hojsgaard *et al.* 2009
Paspalum stellatum	*Killeen, T.J. 2487*	Bolivia	SCZ – Ñuflo de Chávez	500	–16	–62	20	Killeen 1990
Paspalum stellatum	*Killeen, T.J. 2477*	Bolivia	SCZ – Ñuflo de Chávez	500	–16,133	–62,083	32	Killeen 1990
Paspalum stellatum	*Valls, J.F.M. et al. 11689*	Brazil	GO – Acreúna		–17,407	–50,403	52	Honfi *et al.* 1990
Paspalum stellatum	*Valls, J.F.M. et al. 10364*	Brazil	MS – Corumbá	87	–19,873	–57,31	32	Pozzobon *et al.* 2000
Paspalum stellatum	*Quarín, C.L. et al. 3716*	Brazil	RS – São Pedro do Sul		–29,636	–54,289	32	Honfi *et al.* 1990
Paspalum stellatum	*Rua, G.H. et al. 192*	Argentina	MSN – Candelaria		–27,42	–55,6	32	This study
Paspalum stellatum	*BAA 28207 (Honfi, A.I. et al. 1245)*	Argentina	MSN – San Ignacio		–27,283	–55,568	32	This study
Paspalum stellatum	*BAA 28209 (Honfi, A.I. et al. 1537)*	Argentina	MSN – San Ignacio	114	–27,323	–55,535	32	This study
Paspalum stellatum	*BAA 28208 (Honfi, A.I. et al. 1253)*	Argentina	MSN – Candelaria		–27,483	–55,478	20	This study
Paspalum stellatum	*Rua, G.H. 1045*	Bolivia	SCZ – Ñuflo de Chávez	481	–16,166	–62,026	20	This study
Paspalum stellatum	*Rua, G.H. 1048*	Bolivia	SCZ – Ñuflo de Chávez	478	–16,013	–62,019	20	This study
Paspalum stellatum	*Rua, G.H. 1052*	Bolivia	SCZ – Ñuflo de Chávez	485	–16,162	–62,065	20	This study
Paspalum stellatum	*Rua, G.H. 1055*	Bolivia	SCZ – Ñuflo de Chávez	430	–16,374	–62,506	20	This study
Paspalum stellatum	*Rua, G.H. 1057*	Bolivia	SCZ – Ñuflo de Chávez	452	–16,393	–62,504	20	This study
Paspalum stellatum	*Rua, G.H. 1056*	Bolivia	SCZ – Ñuflo de Chávez	468	–16,381	–62,505	32	This study
Paspalum stellatum	*Rua, G.H. 1058*	Bolivia	SCZ – Ñuflo de Chávez	463	–16,394	–62,504	32	This study
Paspalum stellatum	*Rua, G.H. et al. 1031*	Brazil	DF – Sobradinho II	885	–15,544	–47,835	30	This study
Paspalum stellatum	*Rua, G.H. et al. 902*	Brazil	DF – Sobradinho II	912	–15,577	–47,937	32	This study
Paspalum stellatum	*Rua, G.H. et al. 903*	Brazil	DF – Sobradinho II	914	–15,574	–47,944	32	This study
Paspalum stellatum	*Rua, G.H. et al. 933*	Brazil	GO – Acreúna	530	–17,407	–50,403	52	This study
Paspalum stellatum	*Rua, G.H. et al. 937*	Brazil	GO – Acreúna	531	–17,407	–50,404	52	This study
Paspalum stellatum	*Rua, G.H. et al. 932*	Brazil	GO – Acreúna	526	–17,407	–50,403	56	This study
Paspalum stellatum	*da Silva, A.S. et al. 255*	Brazil	GO – Alto Paraiso	1403	–14,062	–47,504	44	This study
Paspalum stellatum	*da Silva, A.S. et al. 256*	Brazil	GO – Alto Paraiso	941	–13,849	–47,303	44	This study
Paspalum stellatum	*da Silva, A.S. et al. 257*	Brazil	GO – Alto Paraiso	797	–13,875	–47,263	44	This study
Paspalum stellatum	*da Silva, A.S. et al. 258*	Brazil	GO – Alto Paraiso	797	–13,875	–47,263	44	This study
Paspalum stellatum	*da Silva, A.S. et al. 259*	Brazil	GO – Alto Paraiso	1207	–14,141	–47,663	44	This study

Table 1. Continued

Species	Voucher	Country	Locality	Altitude (m)	Latitude	Longitude	2*n*	Reference for chromosome count
Paspalum stellatum	*Rua, G.H. et al. 826*	Brazil	GO – Alto Paraiso	1225	–14,101	–47,499	46	This study
Paspalum stellatum	*Rua, G.H. et al. 990*	Brazil	MT – Cuiabá	192	–15,464	–56,051	20	This study
Paspalum stellatum	*Rua, G.H. et al. 970*	Brazil	MT – Cáceres	210	–15,809	–57,458	32	This study
Paspalum stellatum	*Rua, G.H. et al. 985*	Brazil	MT – Cuiabá	200	–15,455	–56,167	32	This study
Paspalum stellatum	*Rua, G.H. et al. 1019*	Brazil	MT – Nová Nazaré	296	–14,412	–52,228	20	This study
Paspalum stellatum	*Rua, G.H. et al. 1012*	Brazil	MT – Pontal do Araguaia	377	–16,043	–52,375	48	This study
Paspalum stellatum	*Rua, G.H. et al. 1010*	Brazil	MT – Pontal do Araguaia	374	–16,043	–52,374	60	This study
Paspalum stellatum	*Rua, G.H. et al. 973*	Brazil	MT – Porto Estrela	212	–15,666	–57,273	52	This study
Paspalum stellatum	*Rua, G.H. et al. 978*	Brazil	MT – Porto Estrela	212	–15,666	–57,273	60	This study
Paspalum stellatum	*Rua, G.H. et al. 866*	Brazil	MT – Unaí	759	–16,227	–47,286	32	This study
Paspalum stellatum	*Valls, J.F.M. et al. 14306*	Brazil	RS – Rosario do Sul	120	–30,24	–54,795	32	This study
Paspalum stellatum	*Rua, G.H. et al. 552*	Paraguay	Central		–27,323	–55,554	32	This study
Paspalum stellatum	*Rua, G.H. et al. 566*	Paraguay	Cordillera		–25,185	–57,227	32	This study

References (Table 1)**:** For Argentina: COS, Corrientes; ERS, Entre Ríos; MSN, Misiones. For Brasil: DF, Federal District; MT, Mato Grosso; MG, Minas Gerais, SP, São Paulo; GO, Goiás; PR, Paraná; RS, Rio Grande do Sul.

plastid DNA regions [the *trnL* (UAA) intron, the *trnL* (UAA)-*trnF* (GAA) spacer, the *atpB*-rbcL spacer and the *trnG* (UCC) intron] were amplified and sequenced. Twenty accessions of nine taxa of *Paspalum* were included in the study: 11 accessions belonging to different cytotypes of *P. stellatum* plus and one accession each of *P. eucomum*, *P. malmeanum*, *P. schesslii*, *P. almum* Chase, *P. bertonii* Hack., *P. modestum* Mez, *P. palustre* Mez, *P. maculosum* Trin; *Axonopus furcatus* (Flüggé) Hitchc. was included for rooting the cladograms. Voucher information and GenBank accession numbers are provided in Appendix 1.

Protocols for DNA isolation, amplification and sequencing and primer information followed those described in Vaio *et al.* (2005). Both strands were sequenced for all taxa. PCR products were sequenced by Macrogen, Inc. (Korea). Assembly and editing of sequences were performed using the software Chromas Pro 1.34 (Technelysium). Sequences were pre-aligned with the Clustal-W (Thompson, Higgins, & Gibson, 1994) algorithm using BioEdit 7.0.9.0 (Hall, 1999) and the alignment was then adjusted manually. All sequences were deposited in GenBank. Two matrices were assembled for the entire set of taxa: one of them containing all four plastid DNA markers and the other, the ITS sequences.

Phylogenetic analyses

Parsimony analyses were performed using TNT 1.1 (Goloboff *et al.*, 2008). Both matrices were analysed under equal character weights. A heuristic search strategy was adopted, consisting of 1000 random addition sequences followed by TBR swapping, using Wagner trees as starting trees and retaining ten trees each time. Branch support was assessed with 10 000 parsimony jackknifing (JK, Farris *et al.*, 1996), using ten series of random addition sequences, swapped using TBR and retaining two optimal trees per series. Branches with ambiguous support (minimum length = 0) were collapsed.

In addition, a Bayesian analysis of the total evidence matrix was conducted using MrBayes version 3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). We established a molecular model using JModelTest (Posada, 2008) (*atpB-rbcL*, GTR+Γ; *trnG*, HKY+Γ; *trnL*, *trnL-F* and ITS, GTR+Γ+I). We carried out two independent runs of 10 000 000 generations using four Markov chains (one cold and three heated chains), sampling every 1000 generations. Posteriors were analysed after a burn-in of 1000 trees. The program Tracer v.1.4 (Drummond & Rambaut, 2007) was used to examine Bayesian parameters to determine stationarity. The first 50 000 trees in each run were discarded as burn-in and the remaining trees combined. Trees were visualized using TreeView (Page, 1996) and FigTree v1.3.1 (Rambaut, 2009).

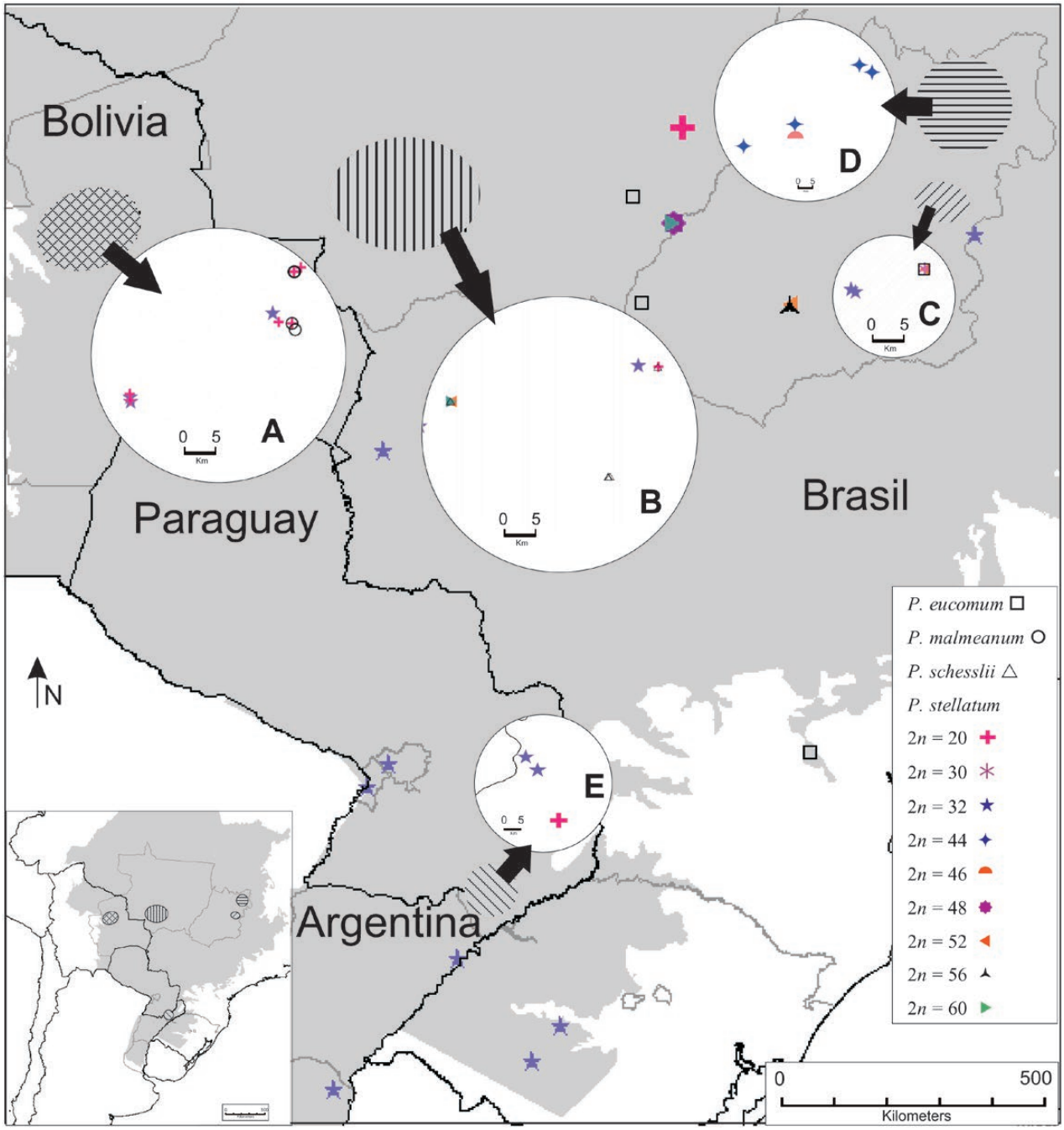

Figure 1. Geographical distribution of the analysed cytotypes of *Paspalum stellatum* and related species in South America. The grey area indicates the general distribution range of *P. stellatum* with $2n = 32$. Circled areas highlight regions with many overlapping points. Possible contact zones between related species and cytotypes: (A) Ñuflo de Chávez, Santa Cruz, Bolivia; (B) Cuiabá, Mato Grosso, Brazil; (C) Sobradinho II, Distrito Federal, Brazil; (D) Alto Paraiso, Goiás, Brazil and (E) Candelaria, Misiones, Argentina.

RESULTS

CHROMOSOME COUNTING, PLOIDY AND GEOGRAPHICAL DISTRIBUTION

Chromosome number determinations were performed for 35 accessions of the *P. stellatum* complex (Table 1). Chromosome numbers of $2n = 20$ (eight accessions), $2n = 32$ (13 accessions) and $2n = 52$ chromosomes (three accessions) were found, in agreement with previous reported counts; several new chromosome numbers ($2n = 30$, 44; 46, 48, 56 and 60) were also found (Table 1, Fig. 2).

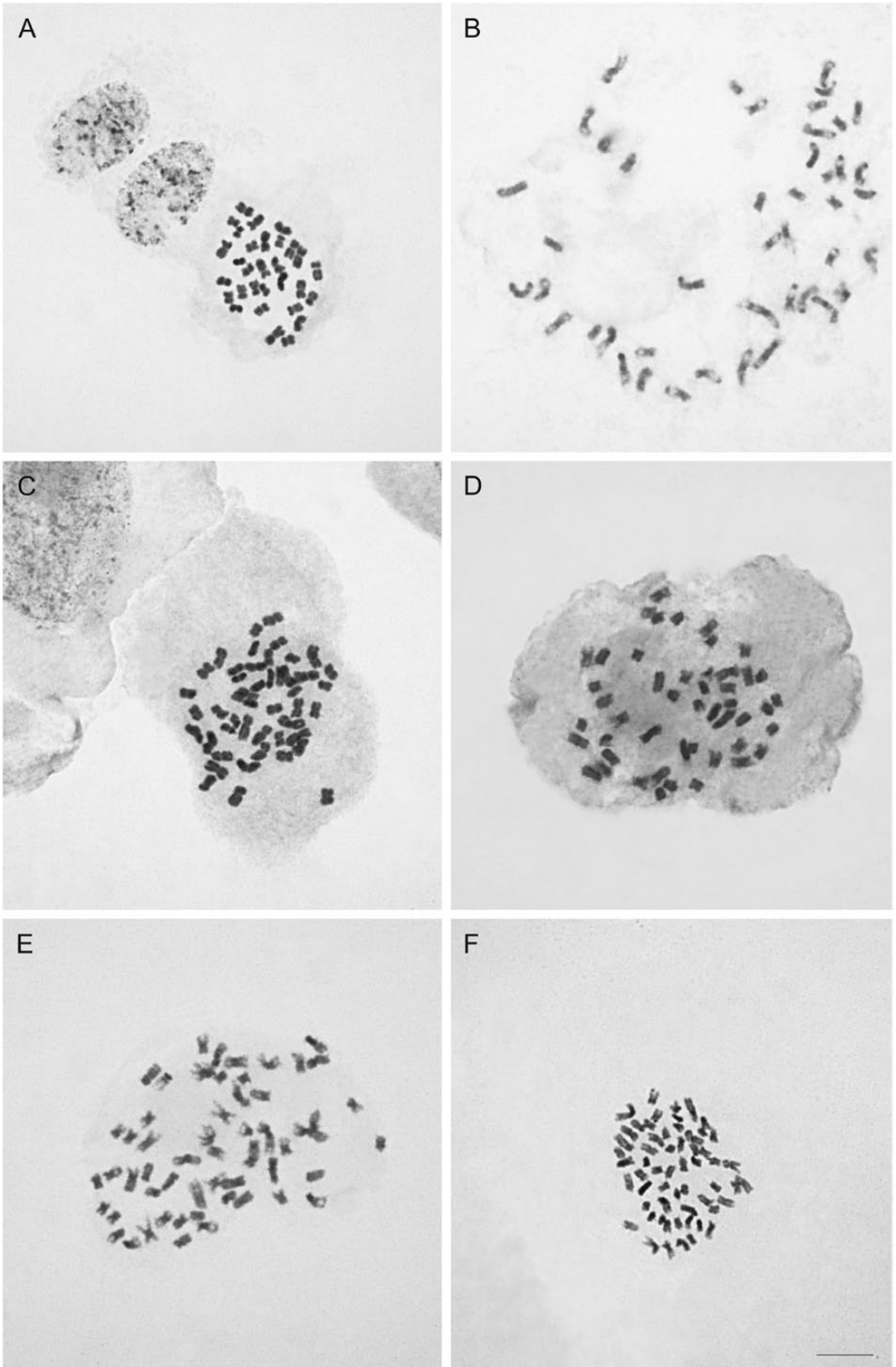

Figure 2. Unusual chromosome counts of *Paspalum stellatum*: A, 2*n* = 30 (*Rua et al. 1031*), DF, Sobradinho II. B, 2*n* = 44 (ASS et al. 259). C, 2*n* = 46 (*Rua et al. 826*), GO, Alto Paraiso. D, 2*n* = 48 (*Rua et al. 1012*), MT, Pontal do Araguaia. E, 2*n* = 56 (*Rua et al. 932*), GO, Acreúna. F, 2*n* = 60 (*Rua et al. 1010*), MT, Pontal do Araguaia. Bar = 5 μm.

When analysing the distribution of the different cytotypes, we identified three regions where diploids ($2n$ = 20) coexist with the cytotype with $2n$ = 32 chromosomes: Ñuflo de Chávez, Santa Cruz, Bolivia (two accessions with $2n$ = 20, *Rua et al. 1055* and *1057*, and two accessions with $2n$ = 32, *Rua et al. 1056* and *1058*; area A, Fig. 1); Cuiabá, Mato Grosso, Brazil (one accession with $2n$ = 20, *Rua et al. 990*, and one accession with $2n$ = 32, *Rua et al. 985*; area B, Fig. 1) and Candelaria, Misiones, Argentina (one accession with $2n$ = 20, *BAA 28208*, and three accessions with $2n$ = 32, *Rua et al. 192*, *BAA 28207* and *BAA 28209*; area E, Fig. 1). In area A, we confirmed the occurrence of *P. malmeanum* $2n$ = 20 (*Rua 1040*) and in area B, we confirmed the occurrence of the presence of *P. schesslii* $2n$ = 12 (*Rua et al. 991*).

The coexistence of rare cytotypes of *P. stellatum* and related species in different combinations was also found in the following localities of the Brazilian cerrado (see Eiten, 1972; Ribeiro & Walter, 1998). Near Sobradinho II, DF, we collected *Rua et al. 1031* with $2n$ = 30 (Fig. 2A). Also, in this location we found *P. stellatum* with $2n$ = 32 (*Rua et al. 902* and *903*) and one accession of *P. eucomum* $2n$ = 32 (*Rua et al. 1124*). In the vicinity of Alto Paraiso, in the Chapada dos Veadeiros, Goiás, the accession *da Silva et al. 255–259* had $2n$ = 44 (Fig. 2B) and the accession *Rua et al. 826* had $2n$ = 46 (Fig. 2C). In Pontal do Araguaia, Mato Grosso, we found populations with $2n$ = 48 (*Rua et al. 1012*, Fig. 2D) and $2n$ = 60 (Rua *et al.* 1010, Fig. 2F). In Porto Estrela, Mato Grosso, accession *Rua et al. 978* had $2n$ = 60, and *Rua et al. 973* had $2n$ = 52; in the same locality one accession of *P. schesslii* with $2n$ = 12 was collected (*Rua et al. 975*). In Acreúna, Goiás, accession *Rua et al. 932* had $2n$ = 56 (Fig. 2E) and *Rua et al. 933* and *Rua et al. 937* had $2n$ = 52.

Phylogenetic analysis

Plastid DNA

The aligned plastid DNA matrix consisted of 2022 characters of which 62 were potentially phylogenetically informative. Parsimony analysis yielded three equally most-parsimonious trees of 113 steps (Supplementary material A).

From the tree topology inferred by Bayesian analysis of the plastid sequences, two major clades can be identified (Fig. 3A), here identified as clade 1 and clade 2. Clade 1 (PP = 1.0; JK = 100) included all accessions of *P. stellatum* with $2n$ = 32 (*Rua et al. 192*, *970* and *1058* and *Valls et al. 14306*) and *P. schesslii* $2n$ = 12 (*Rua et al. 991*), *P. eucomum* $2n$ = 32 (*Rua et al.1124*) and *P. malmeanum* $2n$ = 20 (*Rua et al.1040*). In this clade, *P. stellatum* (*Valls et al. 14306*) and *P. eucomum* (*Rua et al. 1124*) appear to be closely related (PP = 1.0). Clade 2 (PP = 1.0; JK = 86) comprised diploid *P. stellatum* and polyploid accessions with $2n$ = 44 (*da Silva et al. 259*), $2n$ = 48 (*Rua et al. 2012*), $2n$ = 52 (*Rua et al. 937*), $2n$ = 56 (*Rua et al. 932*) and $2n$ = 60 (*Rua et al. 1010*).

Nuclear DNA

The ITS matrix consisted of 652 characters of which 106 were potentially parsimony informative. Parsimony analysis of the ITS data yielded one most-parsimonious tree of 207 steps (Supplementary Material B). Both parsimony and Bayesian analyses yielded two strongly supported clades (Fig. 3B, Supplementary Material B): clade 1 (PP = 0.96; JK = 74) including *P. schesslii* and a subclade grouping *P. eucomum* and *P. malmeanum* (PP = 0.99; JK = 91); and clade 2 (PP = 1.0) including all accessions of *P. stellatum*. In Clade 2, a subclade including the cytotypes from Brazil with $2n$ = 32, 52 and 56 was supported in both analyses (PP = 0.96; JK = 63). Finally, the Bayesian analysis grouped the cytotype from Bolivia with $2n$ = 32 with a Bolivian diploid with $2n$ = 20 (PP = 0.83).

With a few exceptions, there were no major conflicts between the trees yielded by Bayesian and maximum parsimony analyses, in the sense that there were no clades that were strongly supported by one analysis but contradicted by the other. This is shown by including the jackknife support values (JK) for the clades that were shared in both types of analyses, in addition to the associated posterior probabilities (PP) of each clade (Fig. 3).

DISCUSSION

Our results corroborate previous counts for *P. stellatum* (Honfi *et al.*, 1990; Killeen, 1990; Pozzobon, Valls, & Santos, 2000; Sader *et al.*, 2008; Hojsgaard *et al.*, 2009) and also include new unusual counts with different ploidies ($2n$ = 30, 46, 48, 56 and 60) that were found in samples from the Brazilian cerrado. Diploid cytotypes with $2n$ = 20 occur along the western margins of the studied area, in Argentina, Bolivia and Brazil, but were not found in Paraguay. Populations with exclusively $2n$ = 32 were found in large areas of the studied region, including eastern Paraguay, indicating that it is the most widely distributed of all cytotypes analysed. Accessions with $2n$ = 44 were only found in the Chapada dos Veadeiros, Goiás, Brazil, and populations with $2n$ = 52 were found in more restricted geographical areas of the cerrado in Acreúna, Goiás and Porto Estrela, Mato Grosso. Based on these results, it seems that the cytotype $2n$ = 32 of *P. stellatum* is probably the only one that is widespread in the centre of South America.

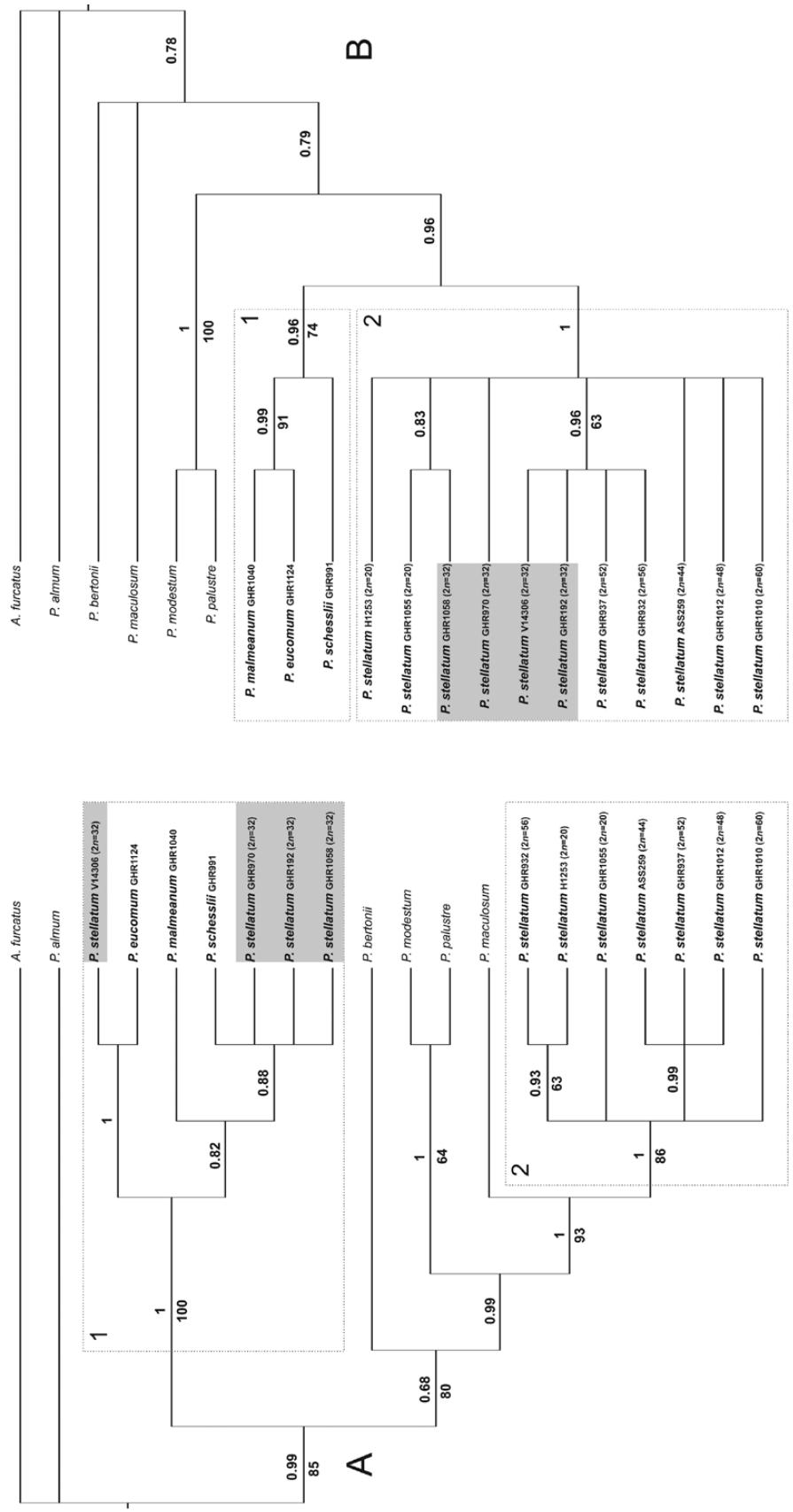

Figure 3. Bayesian analysis of plastid and nuclear sequences. A, Consensus tree obtained from sequences of *atpB-rbcL*, *trnG*, *trnL* and *trnL-F* using a combined dataset. B, Consensus tree obtained from nuclear ITS data. Values above the branches represent posterior probabilities and below the branches jackknife support value. Individuals of *P. stellatum* with $2n = 32$ are highlighted in grey.

Paspalum stellatum is distinguished from the other two related diploid species, *P. malmeanum* ($2n = 20$) and *P. schesslii* ($2n = 12$), by having remarkably wider membranous rachises. Moreover, and unlike *P. schesslii*, *P. malmeanum* has the upper florets narrowly elliptic and firmly attached to the rachilla (Bonasora *et al.*, 2015).

Previous studies have suggested that *P. stellatum* ($2n = 32$) and *P. almum* ($2n = 12$) are closely related (Sader *et al.*, 2008), since the basic number $x = 6$ of *P. almum* is consistent with a hypothesis of allopolyploidy for the origin of *P. stellatum*. However, neither morphology nor the DNA sequence data presented here currently support such a relationship. The recent discovery of *P. schesslii*, which also has $2n = 12$ and is more closely related to *P. stellatum* (Fig. 3), provides a more plausible explanation of the allopolyploid origin for the $2n = 32$ cytotype (see below). Under this scenario, the basic number $x = 6$ would have originated at least twice during the evolution of *Paspalum*.

In a previous study based on cytogenetic data, Bonasora *et al.* (2015) proposed *P. schesslii* ($2n = 2x = 12$) as one of the putative progenitors of the $2n = 32$ cytotype. Our findings are consistent with this hypothesis, since the cytotypes of *P. stellatum* are split into two clades in the plastid DNA analysis, one of which (clade 1) includes all accessions with $2n = 32$ and *P. schesslii*. In the ITS phylogenetic analysis, *P. schesslii*, *P. malmeanum* and *P. eucomum* were placed together as expected (clade 1, PP = 0.96/JK = 74) and all the cytotypes of *P. stellatum* were grouped in the same clade (clade 2, PP = 1, Fig. 3B), indicating that they may all share the same paternal progenitor. Since the plastid DNA is maternally inherited in the majority of angiosperms, including grasses (Giussani *et al.*, 2009; Lawrence & Datwyler, 2016), we can hypothesize that *P. schesslii* could be the putative donor of the maternal genome of the $2n = 32$ cytotype, whereas a diploid cytotype of *P. stellatum* is the presumed paternal progenitor.

Two mechanisms leading to the formation of polyploids in *Paspalum* have been proposed. One of them involves the occasional fertilization of an aposporous embryo sac from a rare apomictic triploid with a normally reduced gamete from a diploid. The other one implies the fertilization of an unreduced gamete from a diploid by a reduced gamete of a naturally occurring tetraploid (e.g. Hörandl & Hojsgaard, 2012; Ortiz *et al.*, 2013; Sartor *et al.*, 2013). Both mechanisms seem unlikely in the case of *P. stellatum* ($2n = 32$), since a triploid cytotype of *P. schesslii* with $2n = 18$ is hitherto unknown and no cytotype with $2n = 40$ chromosomes has ever been found in *P. stellatum* or related species. Our finding of a rare triploid ($2n = 3x = 30$) of *P. stellatum*, however, suggests that a slight variant of the second mechanism could be possible (Ramsey & Schemske, 1998). This would involve a male gamete from *P. stellatum* with 20 chromosomes from a triploid plant with an unreduced female gamete ($2n = 12$) from *P. schesslii*. Nevertheless, we have no information about its fertility and ability to be involved in interspecific crosses.

A third possibility seems more plausible, in which a reduced gamete from *P. schesslii* (i.e. $n = 6$) could have been fertilized by a reduced male gamete from a $2n = 20$ diploid *P. stellatum* (i.e. $n = 10$) giving rise to a 16-chromosome zygote that underwent spontaneous doubling during the first phases of development (Otto & Whitton, 2000; Rieseberg & Willis, 2007; Buggs *et al.*, 2009), in a similar way to that suggested for other allopolyploid species of *Paspalum* (Burson & Bennett, 1972; Burson, 1978; Burson, 1979; Burson & Quarin, 1982; Speranza, 2009).

Contact zones are of special interest since they provide an important source of genetic variation due to the occurrence of heterozygous individuals originating by hybridization between different species or ploidy levels (Burton & Husband, 1999; Halverson *et al.*, 2008). Two contact zones between cytotypes of *P. stellatum* and related species were identified, one of them in the mid-western region of Brazil and the other in eastern Bolivia. In Brazil, the cytotypes $2n = 20$ and $2n = 32$ of *P. stellatum* coexist with *P. schesslii* $2n = 12$. Therefore, the sympatric occurrence of these cytotypes at least provides the opportunity for an allopolyploid origin of the $2n = 32$ cytotype to arise.

Cytotypes with $2n = 30$ and $2n = 32$ chromosomes are also known for the related species *P. eucomum* (Bonasora *et al.*, 2015). The latter taxon perhaps constitutes another polyploid complex like *P. stellatum* and may have originated in a similar way. The fact that one accession of *P. stellatum* and one of *P. eucomum*, both with $2n = 32$ chromosomes, appear together in the plastid DNA phylogenetic is consistent with this hypothesis.

The discovery of several accessions of *P. stellatum* having unusual chromosome numbers from a limited geographical area was unexpected. At the moment, there is no further information to suggest how the rest of the cytotypes have originated, but the available phylogenetic data at least suggest that they were probably formed inside the complex identified as *P. stellatum*.

In summary, diploid cytotypes of *P. stellatum*, *P. malmeanum* and *P. schesslii* are closely related entities that not only share a common ancestor, but also continue crossing and generating interspecific hybrids giving rise to allopolyploid complexes. So far there are few accessions studied, a reason why it is necessary to expand the number of collections and further studies to reveal the genetic and taxonomic status of the different cytotypes.

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Appendix 1. List of the species included in the phylogenetic analysis, country of origin, voucher specimens, herbarium, informal grouping (*sensu* Zuloaga & Morrone, 2005), chromosome number and GenBank accession codes.

Species		Country	Voucher		Herbarium	Group	2*n*	*atpB–rbcL*	*trnG*	*trnL*	*trnL–trnF*	ITS
Axonopus furcatus	(Flüggé) Hitchc.	USA	*Speranza, P. R.*	*s.n.*	CEN			EU627202	EU627280	EU627358	EU627436	
Paspalum almum	Chase	Argentina	*Rua, G. H. & Fernández, J.*	*582*	BAA	*Alma*	12	EU627204	EU627282	EU627360	EU627438	MG969943
Paspalum bertonii	Hack.	Argentina	*Rua, G. H. et al.*	*482*	BAA	*Bertoniana*	20	EU627209	EU627287	EU627365	EU627443	MG969944
Paspalum maculosum	Trin.	Argentina	*Rua, G. H. et al.*	*487*	BAA	*Notata*	20	EU627246	EU627324	EU627402	EU627480	MG969945
Paspalum maculosum	Trin.	Brazil	*Rua, G. H. et al.*	*669*	BAA	*Notata*		-	-	-	-	
Paspalum modestum	Mez	Argentina	*Rua, G. H. et al.*	*146*	BAA	*Plicatula*	20	EU627249	EU627327	EU627405	EU627483	MG969946
Paspalum palustre	Mez	Argentina	*Quarín, C. L.*	*3648*	CTES	*Plicatula*	20	EU627253	EU627331	EU627409	EU627487	MG969947
Paspalum eucomum	Nees ex Trin.	Brazil	*Rua, G. H. et al.*	*1124*	BAA	Subgenus *Ceresia*	32	MG934361	MG963934	MG963921	MG963947	MG969949
Paspalum malmeanum	Ekman	Bolivia	*Rua, G. H.*	*1040*	BAA	Subgenus *Ceresia*	20	MG934360	MG963933	MG963920	MG963946	MG969948
Paspalum schesslii	Bonasora & Rua	Brazil	*Rua, G. H. et al.*	*991*	BAA, UnB, CEN	Subgenus *Ceresia*	12	MG934362	MG963935	MG963922	MG963948	MG969950
Paspalum stellatum	Humb. & Bonpl. ex Flüggé	Argentina	*Rua, G. H. et al.*	*192*	BAA	Subgenus *Ceresia*	32	EU627269	EU627347	EU627425	EU627503	MG969955
aspalum stellatum	Humb. & Bonpl. ex Flüggé	Argentina	*Honfi, A. I. et al.*	*1253*	BAA	Subgenus *Ceresia*	20	MG934363	MG963936	MG963923	MG963949	MG969951
Paspalum stellatum	Humb. & Bonpl. ex Flüggé	Bolivia	*Rua, G. H.*	*1058*	BAA	Subgenus *Ceresia*	32	MG934367	MG963940	MG963927	MG963953	MG969956
Paspalum stellatum	Humb. & Bonpl. ex Flüggé	Bolivia	*Rua, G. H. et al.*	*1055*	BAA	Subgenus *Ceresia*	20	MG934364	MG963937	MG963924	MG963950	MG969952
Paspalum stellatum	Humb. & Bonpl. ex Flüggé	Brazil	*Valls, J. F. M. et al.*	*14306*	CEN	Subgenus *Ceresia*	32	MG934366	MG963939	MG963926	MG963952	MG969954
Paspalum stellatum	Humb. & Bonpl. ex Flüggé	Brazil	*Rua, G. H. et al.*	*970*	BAA, UnB, CEN	Subgenus *Ceresiaa*	32	MG934365	MG963938	MG963925	MG963951	MG969953
Paspalum stellatum	Humb. & Bonpl. ex Flüggé	Brazil	*Rua, G. H. et al.*	*1012*	BAA, UnB, CEN	Subgenus *Ceresia*	48	MG934371	MG963944	MG963931	MG963957	MG969960
Paspalum stellatum	Humb. & Bonpl. ex Flüggé	Brazil	*da Silva, A.S. et al.*	*259*	BAA, UnB, CEN	Subgenus *Ceresia*	44	MG934368	MG963941	MG963928	MG963954	MG969957
Paspalum stellatum	Humb. & Bonpl. ex Flüggé	Brazil	*Rua, G. H. et al.*	*937*	BAA, UnB, CEN	Subgenus *Ceresia*	52	MG934369	MG963942	MG963929	MG963955	MG969958
Paspalum stellatum	Humb. & Bonpl. ex Flüggé	Brazil	*Rua, G. H. et al.*	*1010*	BAA, UnB, CEN	Subgenus *Ceresia*	60	MG934372	MG963945	MG963932	MG963958	MG969961
Paspalum stellatum	Humb. & Bonpl. ex Flüggé	Brazil	*Rua, G. H. et al.*	*932*	BAA, UnB, CEN	Subgenus *Ceresia*	56	MG934370	MG963943	MG963930	MG963956	MG969959

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary material. Parsimony analyses of plastid and nuclear sequences. A, Consensus tree obtained from sequences of *atpB-rbcL*, *trnG*, *trnL* and *trnL-F* using a combined dataset. B, Consensus tree obtained from nuclear ITS data. Values below the branches jackknife support value. Nodes with weak support (< 0.60) were collapsed.