



Microsporogenesis in *Paspalum conspersum* Schrad. (Virgata group) with different ploidy levels

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ABSTRACT. Knowledge about the cytology and reproductive behavior of a species is indispensable for hybridization programs. This is especially true for species belonging to the genus *Paspalum*, among which apomixis and a wide range of ploidy levels are frequently found. *Paspalum conspersum* Schrad. is a robust and warm-season perennial bunchgrass native to South America. Previous studies have indicated that both tetraploid and hexaploid races exist in this species; however, only information related to tetraploids has been applied to another taxon. In this study, a cytological investigation in two Brazilian accessions collected in different regions revealed tetraploidy in the accession BRA-012823 ($2n = 4x = 40$), with chromosome pairing in bivalents and normal meiosis and tetrad formation, and pentaploidy ($2n = 5x = 50$) in the accession BRA-022748, which presented total asynapsis. In this latter accession, 50 univalents could be scored at diakinesis. After alignment at the metaphase plate, sister chromatids segregated to the poles. Only one meiotic division (equational) occurred, and after

cytokinesis, 100% of the dyads that formed had $2n$ microspores. The meiotic behavior during microsporogenesis, which showed 10 delayed univalents to reach the metaphase plate, suggests that this accession is a recent natural hybrid constituted by a parental genome with 40 chromosomes and another with 10 chromosomes. The potential usage of these accessions in *Paspalum* breeding has been discussed.

Key words: Meiosis; Poaceae; Interspecific hybridization; Pentaploidy; $2n$ gamete

INTRODUCTION

Paspalum is a large and heterogeneous genus of the Poaceae, which includes economically important forage and turf grass cultivars. With few exceptions, *Paspalum* species are native to the tropical and subtropical regions of the Americas (Chase, 1929). The genus presents an immense diversity that extends from the more obvious traits, such as general morphology and growth habits, to its regions of adaptation (Burson, 1997).

In spite of the progress achieved thus far in understanding the cytological behavior of several *Paspalum* species, little or no information is available regarding the basic cytological composition of many other species of this genus. During the past few years, several accessions of different species with forage and turf potential have been selected from a group of 300 accessions collected in Brazil (Batista and Regitano Neto, 2000), including two accessions of *Paspalum conspersum* Schrad. (BRA-012823 and BRA-022748). *P. conspersum*, also known as “scattered paspalum”, is a leafy, robust, warm season perennial bunchgrass native to South America; several cytological studies have been performed on this species (see Table 1).

To date, chromosome counts of $2n = 4x = 40$ and $2n = 6x = 60$ have been attributed to *P. conspersum* strains collected in Argentina, Bolivia, Brazil, and Paraguay. Accurate checking of passport data of the accessions cited by distinct authors (Table 1) confirms the conclusion of Pozzobon et al. (2000) that references to $2n = 40$ in this species can be traced to ill-identified specimens of *P. regnellii* Mez.

Tetraploids mentioned by Fernandes et al. (1974) were collected, maintained, and analyzed in Rio Grande do Sul, the southern most Brazilian State, in the framework of the S3-Cr-11 binational project, which was conducted from 1961 to 1966 to improve knowledge on the natural grasslands of Southern Brazil (Barreto, 1963). Seed samples of other accessions from the same live plant collection were sent to the United States Department of Agriculture, a project co-sponsor. These accessions were studied by Burson and Bennett (1976), at which point accessions of *P. regnellii* were mistakenly identified as *P. conspersum*, and such taxonomic uncertainties persisted for a long time, even leading Renvoize (1988) to formally designate *P. regnellii* as a synonym of *P. conspersum*.

Both species are currently accepted, and methods for their morphological differentiation have been summarized by Zuloaga and Morrone (2005). Phylogenetic distance was clarified in a recent study, where *P. conspersum* remained in the Virgata clade, while *P. regnellii* was part of a different clade, Macrophylla (Giussani et al., 2009). Valls and Oliveira (2012) did not list *P. conspersum* for Rio Grande do Sul State in their contribution for the genus for the online Flora of Brazil database. A reference to $2n = ca. 40$ chromosomes for a *P. conspersum* strain from Bolivia (Killeen, 1990) has been reevaluated. The count of $2n = 4x = 40$ was confirmed, but the voucher was reassigned to *P. plenum* Chase (Norrman et al., 1994).

Table 1. Previous cytological information on plant materials identified as *Paspalum conspersum*.

Original identification	Institutional code	2n	Country	State, Province or Department	Municipality	Source
NF 49	-	40	Brazil	Rio Grande do Sul	[Depressão Central region]	Fernandes et al., 1974
NF 65	-	40	Brazil	Rio Grande do Sul	[Depressão Central region]	Fernandes et al., 1974
[79]	PI 303963	40	Brazil	Rio Grande do Sul	São Francisco de Paula	Burson and Bennett, 1976
NF 617	PI 303964	40	Brazil	Rio Grande do Sul	[Planalto Médio region]	Burson and Bennett, 1976
NF 822	PI 303965	40	Brazil	Rio Grande do Sul	[Campanha region]	Burson and Bennett, 1976
[36]	PI 310056	40	Brazil	Rio Grande do Sul	[Planalto Médio region]	Burson and Bennett, 1976
Ki 2317	-	ca. 40	Bolivia	Santa Cruz, Nufflo de Chavez	Concepción	Killeen, 1990; 2n = 40 in Normmann et al., 1994, under <i>P. plenum</i>
Q 3367	-	60	Argentina	Corrientes	Corrientes	Quarín, 1977
Q 3589	-	60	Argentina	Corrientes	Corrientes	Quarín and Hanna, 1980
V/Sv 10312	BRA-010260	60	Brazil	Mato Grosso do Sul	Miranda	Pozzobon and Valls, 1987; Adamowski et al., 2005
VQFdsV 11819 = Q 4018	BRA-012661	60	Brazil	Mato Grosso do Sul	Ivinhema, near Amandina	Honfi et al., 1990
VQFdsV 11868	BRA-012831	60	Brazil	Mato Grosso do Sul	Itaquiraí [not Igatuemi, as cited]	Pozzobon et al., 2000
VGaRoSv 12542	BRA-017981	60	Brazil	Mato Grosso	Santa Teresinha	Pozzobon et al., 2000
VGaRoSv 12625	BRA-018104	60	Brazil	Mato Grosso	Sorriso	Pozzobon et al., 2000
Contaminant	BRA-022501	60	Brazil	[São Paulo] (Adventive)	[Cultivated in São Carlos]	Pagliari et al., 2001
Contaminant	BRA-022519	60	Brazil	[São Paulo] (Adventive)	[Cultivated in São Carlos]	Pagliari et al., 2001
Gh 572	-	60	Paraguay	Cordillera		Pozzobon et al., 2008
Hj 175	-	60	Argentina	Misiones	General San Martín	Hojsgaard et al., 2009
Hj 194	-	60	Argentina	Misiones	San Ignacio	Hojsgaard et al., 2009

Fd = M.S. França-Dantas; Ga = M.L. Galgari; Gh = G.H. Rua; Hj = D. Hojsgaard; J = L. Jank; Ki = T.J. Killeen; Q = C.L. Quarín; Ro = D.M.S. Rocha; Sv = G.P. Silva; V = J.F.M. Valls. [] = Collecting numbers not linked to collector names. BRA = Brazilian germplasm accession code; NF = Live Plant Collection of Secretaria da Agricultura, Rio Grande do Sul, Brazil/Project S3-Cr-11; PI = USDA Plant Inventory number.

Therefore, all previous chromosome counts correctly assigned to *P. conspersum* populations correspond to $2n = 6x = 60$ (Table 1). They refer to plants collected in the continental Argentinian provinces of Corrientes and Misiones, in the Cordillera Department of Paraguay, as well as in Brazilian sites north of the Tropic of Capricorn. Meiotic and embryo sac studies of some of these hexaploid strains have consistently shown normal pairing as 30 II, with none or very scarce irregularities at segregation, production of normal embryo sacs, and high seed set under both open- and self-pollinated conditions, indicating a sexual, self-compatible mode of reproduction (Quarín and Hanna, 1980; Pozzobon and Valls, 1987; Pagliarini et al., 2001; Adamowski et al., 2005).

However, incorporation of additional accessions of *P. conspersum* to agronomic trials in the *Paspalum* gene bank of Embrapa Cattle - Southeast has focused on two peculiar strains collected in Mato Grosso do Sul State, one of which shows $2n = 4x = 40$, while the other shows $2n = 5x = 50$. In the present study, we report aspects of microsporogenesis of these tetraploid and pentaploid accessions and speculate on their importance and potential applications for the development of new cultivars.

MATERIAL AND METHODS

Two native Brazilian accessions of *P. conspersum*, collected in June 1988 by J.F.M. Valls, C.L. Quarín, M.S. França-Dantas, and G.P. Silva in the State of Mato Grosso do Sul, were analyzed. BRA-012823 (field number VQFdSv 11855) was originally collected at Porto Santo Antônio, near the Paraná River, in the municipality of Itaquiraí. BRA-022748 (VQFdSv 11894) was collected just north of Aral Moreira, along the frontier road that parallels the dry Brazil/Paraguay border. Both accessions are maintained in the *Paspalum* germplasm collection at Embrapa Cattle - Southeast, located in São Carlos, São Paulo State, Brazil.

Young inflorescences were collected and fixed in 3:1 ethyl alcohol:acetic acid for 24 h at room temperature, transferred to 70% aqueous alcohol solution (v/v), and stored in a freezer until evaluated. Meiocytes were obtained by squashing anthers in a drop of 1% propionic-carmin. Chromosome numbers were counted in meiocytes at diakinesis. All meiotic phases were evaluated under light microscopy and meiotic abnormalities were scored. A total of 952 and 11,151 meiocytes were evaluated in the accessions BRA-022748 and BRA-012823, respectively. Photomicrographs were taken using an Olympus microscope (CX30) and analyzed with the AnalySIS getIT software.

RESULTS

Chromosome numbers evaluated at diakinesis in both accessions revealed tetraploidy ($2n = 4x = 40$) in the accession BRA-012823 and pentaploidy ($2n = 5x = 50$) in the accession BRA-022748. Meiotic behavior in the tetraploid accession was quite regular (Table 2) with a few irregularities observed in chromosome segregation (Figure 1). At diakinesis, chromosomes paired exclusively as bivalents (Figure 1a).

Complete asynapsis was detected in all meiocytes of the pentaploid accession BRA-022748 (Table 3). The meiosis in this accession was completely irregular. Fifty univalents were scattered throughout the cytoplasm at late diakinesis (Figure 2a and b). At the 1st meiotic metaphase, one genome with 40 univalents congregated at the equatorial plate, and the other, constituted by 10 univalents, was delayed in reaching the plate relative to the 1st (Figure 2c).

After a certain time, both genomes reached the equatorial plate, forming a unique and typical metaphase plate (Figure 2d). In some meiocytes, the 10 univalents were allocated in an independent metaphase plate (Figure 2e). In anaphase, sister chromatids segregated to the opposite poles (50:50) (Figure 2f), giving rise to a normal telophase (Figure 2g) that, after cytokinesis (Figure 2h), originated a dyad of microspores (Figure 2i). Only one meiotic division (equational) occurred, and two $2n$ microspores were formed.

Table 2. Meiotic behavior in the tetraploid ($2n = 4x = 40$) accession of *Paspalum conspersum* (BRA-012823).

Phase	Number of meiocytes	Meiotic behavior	% abnormal meiocytes
Diakinesis	30	20 bivalents	0.0
Metaphase I	224	Precocious chromosome migration to the poles and non-oriented bivalents	1.64
Anaphase I	58	-	0.0
Telophase I	195	Micronucleus	2.56
Prophase II	152	Micronucleus	1.31
Metaphase II	126	-	0.0
Anaphase II	2	-	0.0
Telophase II	128	Micronucleus	2.34
Tetrad	236	Micronucleus	2.12
Total	1151		

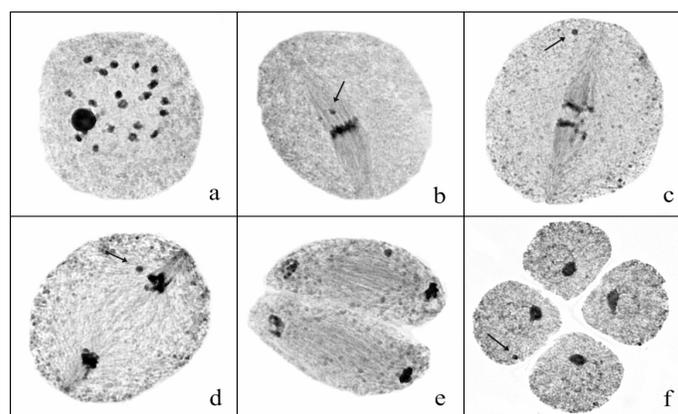


Figure 1. Aspects of microsporogenesis in the tetraploid accession of *Paspalum conspersum* (BRA-012823). **a.** Diakinesis with 20 bivalents. **b.** Metaphase I with precocious chromosome migration to the pole (arrow). **c.** Anaphase I with a non-oriented bivalent (arrow). **d.** Early telophase I with a micronucleus (arrow). **e.** Telophase II with a micronucleus. **f.** Tetrad with a micronucleus (arrow).

Table 3. Meiotic behavior in the pentaploid ($2n = 5x = 50$) asynaptic accession of *Paspalum conspersum* (BRA-022748).

Phase	No. of meiocytes
Diakinesis with 50 univalents	141
Metaphase with a non-congregated genome	96
Metaphase with 50 congregated univalents	249
Normal anaphase	42
Normal telophase	146
Normal cytokinesis after telophase	52
Dyad	226
Total	952

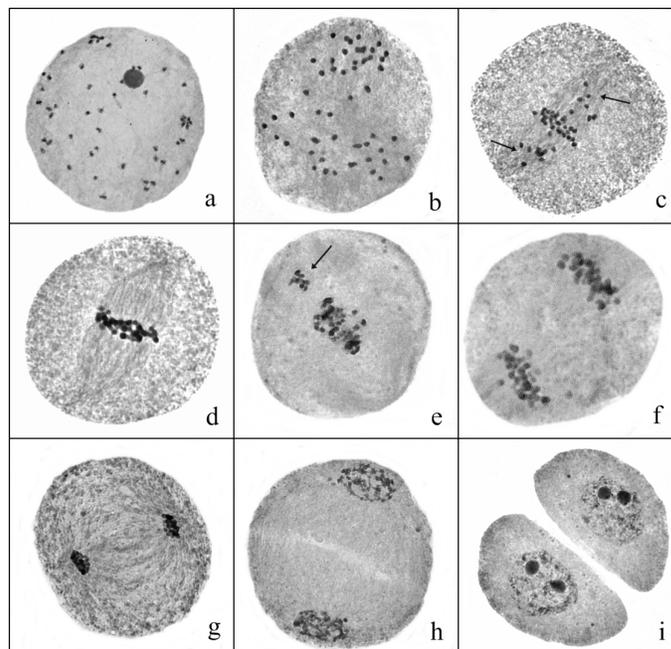


Figure 2. Aspects of microsporogenesis in the pentaploid accession of *Paspalum conspersum* (BRA-022748). **a.** Meiocyte in diakinesis with 50 univalents. **b.** Meiocyte in late diakinesis with 50 univalents. **c.** Meiocytes in metaphase showing one genome congregated at the metaphase plate (40 univalents) and the other with scattered univalents (arrows). **d.** Metaphase with both genomes congregating at the plate. **e.** Metaphase with one genome arranged in a small metaphase plate (arrow). **f.** Normal anaphase. **g.** Normal telophase. **h.** Cytokinesis after telophase. **i.** Dyad with two $2n$ microspores.

DISCUSSION

Cytogenetics of *P. conspersum*

Polyploidy, from triploidy to 16-ploidy, is found in approximately 80% of *Paspalum* species (Quarín, 1992), among which the most frequent chromosome set is $2n = 4x = 40$, and more than 70% of the listed polyploids reproduce by apomixis (Bonilla and Quarín, 1997). Previous tetraploid ($2n = 4x = 40$) reports for *P. conspersum*, associated with regular meiosis and sexual reproduction (Fernandes et al., 1974; Burson and Bennett, 1976), do not, in fact, refer to this species. On the contrary, hexaploidy ($2n = 6x = 60$) has been most commonly reported for true representatives of the species (Quarín, 1977; Quarín and Hanna, 1980; Pozzobon and Valls, 1987; Honfi et al., 1990; Pagliarini et al., 2001; Adamowski et al., 2005; Pozzobon et al., 2000, 2008; Hojsgaard et al., 2009). The 60-chromosome race of *P. conspersum* analyzed by Quarín and Hanna (1980) showed regular meiosis, with primarily bivalent pairing and a low percentage of univalents and quadrivalents. Chromosome distribution was regular at anaphase I and at anaphase II. Similar results were obtained by Pozzobon and Valls (1987), Pagliarini et al. (2001), and Adamowski et al. (2005).

Tetraploidy and pentaploidy were here described for the 1st time for typical representatives of *P. conspersum*. Tetraploidy is the most common pattern in *Paspalum*, while pentaploidy has been reported in only a few species, such as *P. minus* E. Fourn. (Bonilla and Quarín, 1997), *P. alcalinum* Mez (Burson, 1997), *P. dilatatum* Poir. (Mehra and Chaudhary, 1981; Burson et al., 1991; Pozzobon et al., 2000), *P. arundinellum* Mez (Hojsgaard et al., 2009), and *P. notatum* Flüggé (Tischler and Burson, 1995).

Genomes in polyploid accessions of *P. conspersum*

Previous considerations by Burson and Bennett (1976) on genomes of tetraploid accessions of *P. conspersum* must be left aside, as they actually apply to *P. regnellii*. Dealing with a true representative of *P. conspersum*, Quarín and Hanna (1980) hypothesized that the prevalent bivalent pairing at metaphase I and the regular 30:30 chromosome distribution at anaphase I indicated that the 60-chromosome race of *P. conspersum* should be an allopolyploid with very little homology, if any, between its three genomes. The authors suggested that these could have arisen from an unreduced egg of an allotetraploid fertilized by reduced pollen of an autotetraploid species. Yet, another possible origin could be the normal fertilization of a reduced egg from an allotetraploid species by pollen of a diploid species, thus giving rise to a triploid hybrid, followed by further duplication of its chromosomes, thus producing an amphidiploid with 60 chromosomes.

The data obtained in the present study with the pentaploid accession (BRA-022748) of *P. conspersum* showed 40 univalents congregating simultaneously at the metaphase plate and 10 dispersed univalents, suggesting the presence of at least two genomes. In the pentaploid accessions of *Paspalum* analyzed so far, meiosis was reported to be irregular, with chromosomes associated as uni-, bi-, tri-, and quadrivalents (Bonilla and Quarín, 1997; Burson, 1997). In a pentaploid plant of *P. alcalinum*, Burson (1997) suggested that it probably originated from a cross between tetraploid (unreduced egg) and diploid cytotypes (haploid sperm), where the female parent was an apomictic tetraploid and the male was a sexual diploid. In this species, members of the extra genome are present as either univalents or trivalents. In the trivalents, a haploid chromosome was synapsed with one of the members of a bivalent from the female parent. This synapsis represents homology between chromosomes of the haploid genome and a member of one genome of the tetraploid parent. In the present accession (BRA-022748) of *P. conspersum*, the absence of trivalents could indicate that the haploid genome is not homologous to the other genome.

Asynapsis and hybridization

Chromosome pairing during prophase plays a critical role in the sequence of meiotic events. The success of chromosome pairing during the early stages of the 1st meiotic division will affect the viability of the gametes formed (Visser et al., 1999). Synaptic mutants are common in several higher plant families, inclusive of Poaceae (Koduru and Rao, 1981; Singh, 2003). These mutants, which arise mostly spontaneously, may result from natural and artificial interspecific hybridization or might be induced by mutation (Ramanna and Jacobsen, 2003; Singh, 2003). Asynapsis was suggested to have occurred in a hexaploid accession of *P. jesuiticum* Parodi collected in the State of Rio Grande do Sul - Brazil (Fernandes et al., 1974), but details of meiotic behavior were not provided. Asynapsis was also reported in an apomic-

tic tetraploid accession of *P. secans* Hitchc & Chase (Snyder, 1961). Desynapsis, on the other hand, has been reported to occur in *P. conjugatum* P.J. Bergius, *P. dilatatum*, *P. longiflorum* Retz., and *P. orbiculare* G. Forst. (Mehra and Chaudhary, 1981).

The meiotic behavior of the new tetraploid, BRA-012823, is suggestive of a normal, sexual mode of reproduction, whereas that of the pentaploid BRA-022748 accession is typical of hybrids recently formed in nature, characterized by a dissimilar parental genome meiotic rhythm. This phenomenon has been found in one accession of *P. subciliatum* Chase (Adamowski et al., 1998), and is widely reported in species of the genus *Brachiaria* (Trin.) Griseb., which is presently merged (Shirasuna, 2012) with the circumscription of *Urochloa* P. Beauv. (Ricci et al., 2010). Several polyploids were formed in the past by genome duplication events occurring millions of years ago (paleopolyploids), but according to Adams and Wendel (2005), polyploidy is an active and ongoing process; the meiotic behavior of the *P. conspersum* accessions analyzed here reinforces this proposition.

2n gamete formation and the breeding of *Paspalum* genotypes

Equational chromatid division, such as that observed here in accession BRA-022748 of *P. conspersum*, has been observed in synaptic mutants of several species (Ramanna, 1983), including species of the *Paspalum* genus (Snyder, 1961; Mehra and Chaudhary, 1981; Bonilla and Quarín, 1997); dyads generating two 2n pollens were reported in these synaptic mutants.

The incidence of 2n gametes is frequent in the plant kingdom, and their occurrence has been reported in species of many families, including Poaceae (Veilleux, 1985); they result from modified meiosis affecting micro- and megasporogenesis. The absence of cytokinesis leading to 2n gamete formation has also been reported in several *Paspalum* species (Pagliarini et al., 1999).

Spontaneous polyploidy is an important phenomenon, both for evolution and for the development of new cultivars. The two main modes of polyploidization recognized are asexual polyploidization, through somatic doubling of the chromosome complement, and sexual polyploidization, through the formation and functioning of 2n gametes (Harlan and de Wet, 1975; Veilleux, 1985; Bretagnolle and Thompson, 1995), although the latter seems to be predominant in nature.

In the pentaploid accession of *P. conspersum* studied in the present study, 100% of the gametes presented the somatic chromosome number (2n gametes). This is unusual, since even in species or genotypes disposed to 2n gamete formation, the frequency of 2n gametes is generally variable. Veilleux (1985) listed several plant species for which the range of 2n pollen production varied from 0.05 to 99%.

The importance of meiotic mutants to breeding has been emphasized previously, particularly with respect to the potential usefulness of 2n gametes formed by synaptic mutants in transmitting maximal amounts of heterozygosity. In fact, fertile 2n gametes were reported in synaptic mutants of different species (Ramanna, 1983). Bretagnolle and Thompson (1995) listed more than 10 abnormal mechanisms leading to 2n gamete formation. The genetic constitution of 2n gametes falls into two general categories, namely, the 1st division restitution (FDR) and the 2nd division restitution (SDR) (Ramanna and Jacobsen, 2003). If the 2n gamete contains one sister chromatid of each replicated chromosome, it is an FDR 2n gamete. If the 2n gamete includes both sister chromatids of one of the homologous chromosomes, then it is an SDR 2n gamete. The process observed in the accession BRA-022748 *P. conspersum*, in which

asynapsis was accompanied by separation of the univalent sister chromatids at anaphase followed by cytokinesis after telophase, resulted in an FDR 2n gamete.

Sexual polyploidization can be successfully employed both for increasing the allelic diversity and for transferring useful traits from wild-related species into cultivated varieties. In the case of allopolyploids specifically, introgression can be achieved through recombination as a result of crossing-over between alien chromosomes, as well as from the addition of alien chromosomes (Ramanna and Jacobsen, 2003).

High efficiency (100%) in 2n gamete production could be of particular importance for *Paspalum* breeding, for which intra- and interspecific hybridization are both desired. However, as suggested by their meiotic behavior, the pentaploid accession most likely originated from recent natural hybridization, and the genomes involved in its constitution have not yet been identified. Therefore, more studies are needed before it can be effectively used in the breeding program. Another factor impairing its use in breeding is apomixis, which is frequently associated with polyploidy (Quarín, 1992); therefore, sexual counterparts with identical ploidy levels are necessary for hybridization. Sexuality can be found among tetraploid accessions presenting regular meiosis, and was previously reported in strains that were then considered to represent *P. conspersum* (Fernandes et al., 1974; Burson and Bennett, 1976). Unfortunately, such data are valid for a related species, which was initially considered as another member of the same informal group *Virgata*, but is now understood to be phylogenetically more distant. In this context, the finding of a true *P. conspersum* strain (BRA-012823) showing the tetraploid level is promising. Although the mode of reproduction was not directly evaluated in the accession BRA-012823 in the present study, its high meiotic stability suggests that it may reproduce sexually.

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