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Sexual diploid and apomictic tetraploid races in *Thrasya petrosa* (Gramineae)

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Abstract. *Thrasya petrosa* (Trin.) Chase is the most widespread species of a grass genus indigenous to the New World. Genetic systems in diploid ($2n = 2x = 20$) and tetraploid ($2n = 4x = 40$) races of *T. petrosa* were investigated. The diploid race exhibited embryological development typical of sexual reproduction, but failed to produce seed because of self-incompatibility, whereas the tetraploid showed embryological pathways characteristic of facultative apomixis. Consequently, some ovules showed a normal meiotic embryo sac, others had one to several aposporous sacs, and, finally, some ovules had one or more aposporous sacs beside the meiotic one. A uniform progeny test assisted by molecular markers confirmed that the main reproductive mode for the tetraploid race was apomixis, despite some sexual reproductive structures observed by cytoembryological analyses. The chromosome pairing patterns at meiosis suggested that autoploidy was the genetic origin of the tetraploid races of *T. petrosa*. In addition, the close relationship between *Thrasya* Kunth and *Paspalum* L. previously supported by phylogenetic analyses is further sustained by the particular genetic system shared by the two genera. The system involves co-specific sexual self-incompatible diploids and apomictic, pseudogamous and self-compatible polyploids.

Introduction

Thrasya Kunth (Gramineae) is a neotropical grass genus comprising fewer than 20 species. Its distribution extends from Mexico to Paraguay, with an apparent epicentre in northern Brazil, Venezuela and Guyana. *Thrasya petrosa* (Trin.) Chase is the most widespread within the group, extending from Costa Rica to Paraguay (Burman 1985).

According to morphological studies, there is a close relationship between the genus *Thrasya* and both the subgenus *Ceresia* and the informal group Decumbentes of the genus *Paspalum* L. (Burman 1985; Nicora and Rúgolo de Agrasar 1987).

The genus *Thrasya* has a base chromosome number of $x = 10$. Diploid, tetraploid and hexaploid forms have been reported for *T. petrosa* (Pohl and Davidse 1971; Davidse and Pohl 1974; Norrmann *et al*. 1994). Different ploidy levels among individuals of a single species are frequently observed in *Paspalum* species. Several species of *Paspalum* have diploid and tetraploid cytotypes. The diploid representatives reproduce sexually and are self-incompatible, whereas the cospecific tetraploid individuals are characterised by aposporous apomixis, pseudogamy and self-compatibility (Quarin 1992). Data from cytogenetic analyses suggested an autoploid origin of tetraploid *P. notatum* Flügge (Forbes and Burton 1961; Quarin *et al*. 1984) and tetraploid *P. rufum* Nees ex Steud. (Quarin *et al*. 1998), two species with diploid sexual counterparts. In addition, Pupilli *et al*. (1997) demonstrated the existence of tetrasomic inheritance in tetraploid races of *P. simplex* Morong, and Stein *et al*. (2004) reported polysomic inheritance in *P. notatum*, corroborating autoploid origin for this species. However, there is no information available about the reproductive systems of any species of *Thrasya*.

The obscure taxonomic boundary between *Thrasya* and *Paspalum* and the existence of different ploidy levels in *T. petrosa* suggest that apomictic reproduction may be involved in the polyploid series recorded for this species.

The purpose of this research was to determine the reproductive system of the diploid and tetraploid forms of *T. petrosa* and to analyse the possible autoploid origin of the tetraploid cytotype.

Materials and methods

Plant material

Seeds of diploid ($2n = 2x = 20$) and tetraploid ($2n = 4x = 40$) *T. petrosa* were provided in 1989 by Timothy Killeen, Missouri Botanical Gardens, St Louis. The collection localities were the following: diploid collection *Killeen 2418*, Bolivia, Department of Santa Cruz, Province of Ñuflo de Chávez, Estancia Viera, 2 km south of Concepción on the road to Lomerío, 16°08′S, 62°05′W, 500 m; tetraploid collection *Killeen 1953*, Bolivia, Department of Santa Cruz, Province of Ñuflo de Chávez, Estancia Salta, 10 km south of Concepción on the road

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to Lomerío, 16°13′S, 62°00′W, 500 m. Duplicate vouchers of both botanical collections are deposited in the herbarium CTES, Corrientes, Argentina. One plant of each collection was cultivated in a field nursery at Corrientes, Argentina.

Meiotic behaviour

Material used to study meiosis was fixed in a 3 : 1 solution (v/v) of absolute ethanol : acetic acid for 24 h and stored in 70% (v/v) ethanol. Pollen mother cells were stained with aceto-carmine and the cells undergoing meiosis were examined with a phase contrast microscope to study meiotic behaviour.

Reproductive system

Embryological analyses as well as observations on pollen and seed fertility were used to determine the method of reproduction. In addition, a progeny test was conducted with the aid of molecular markers to confirm the method of reproduction in the tetraploid.

For embryological analyses, inflorescences at different stages of development were fixed and stored in FAA (70% ethanol : glacial acetic acid : formaldehyde, 18 : 1 : 1). Entire young spikelets were dehydrated in a tertiary butyl alcohol series and embedded in paraffin. Samples were sectioned at 12 µm and stained with safranin and fast green. Preparations were observed under light transmission microscope.

Pollen germination on the stigma surface and tube growth following self-pollination were determined by fluorescence microscopy: pistils were dissected from spikelets 20, 40, 60, 90 and 120 min after pollination, placed in 1 mol L^{-1} NaOH for 15 min, and transferred into 0.1% (w/v) aniline blue solution. Each sample comprised approximately 20 pistils. Penetration of the pollen tubes and their growth up to the micropilar zone were recorded.

Seed set (percentage of spikelets forming caryopses) was determined under self-pollination conditions. Since only one individual plant was grown in the field for each ploidy level (i.e. the diploid and the tetraploid strains), cross-pollination was not possible at the same ploidy level. Immature inflorescences were enclosed in glassine bags to prevent seed loss from spikelet shattering before full maturation of the inflorescences. Mature inflorescences were harvested and each spikelet was examined for seed formation.

DNA fingerprints were used for progeny analysis to support the proposed method of reproduction in tetraploid *T. petrosa*, in addition to the cytoembryological analysis. Random amplified polymorphic DNA (RAPD) markers were used for fingerprinting the tetraploid plant and 10 individuals of its offspring. Genetic differences between the mother plant and its progeny would indicate sexual reproduction, whereas genetic uniformity among the mother plant and the individual progenies would authenticate the apomictic reproductive system assessed by embryological analyses.

DNA extraction and RAPD analysis

Genomic DNA was extracted from some young leaves of each plant according to Martínez *et al*. (2003).

Forty arbitrary decamers (set 5) from the RAPD Primer Synthesis Project of the British Columbia University were assayed. Polymerase chain reactions (PCR) were performed in 25 µL total volume, containing 20 ng genomic DNA, 1 × *Taq* polymerase reaction buffer, 100 µmol dNTPs, 1.5 mmol $MgCl_2$, 1 U *Taq* DNA polymerase (Promega) and 30 ng of primer. Amplifications were carried out by using a Biometra UNO-Thermoblock reference. Cycles began with 1 min at 93°C, followed by 40 cycles of 1 min at 93°C, 1 min at 36°C and 2 min at 72°C, and a final extension of 5 min at 72°C. Amplification products were electrophoresed in 2% w/v agarose gels/1 × TAE (40 mmol Tris, 5 mmol sodium acetate, 0.77 mmol EDTA, pH 8.0) at 40 V for 4 h. Gels were stained with ethidium bromide and fragments were visualised in a transilluminator emitting ultraviolet light. Photographs were taken with a Kodak DC290 digital camera, and the pictures were collected as TIF images and further analysed with the program Adobe PhotoDeluxe Business Edition Version 1.0.

Results

Meiosis

Meiotic chromosome behaviour of the diploid plant showed 10 bivalents at diakinesis and Metaphase I, and chromosomes distributed regularly during Anaphase I. Meiosis in the tetraploid plant was irregular. Univalents, bivalents, trivalents and quadrivalents were observed at Metaphase I (Table 1). In addition, lagging chromosomes were present in 30 of 69 meiocytes (43.5%) observed at Anaphase I. Laggards averaged 3.5 per meiocyte.

Reproductive behaviour

From the diploid plant, 120 ovules at different stages of development were examined. Megasporogenesis was fairly normal: the meiocyte underwent meiosis to produce a linear triad of megaspores because the second meiotic division only occurred in the chalazal member of the dyad. Then, the two micropilar members of the triad degenerated and the chalazal one was the functional megaspore. After several mitotic divisions, the functional megaspore developed the megagametophyte. The mature embryo sac had one egg cell, two synergids, one central cell with two polar nuclei, and >30 antipodal cells per sac. Since the megagametophyte originated from a product of meiosis (the functional megaspore), it was classified as a meiotic embryo sac, which has haploid nuclei. Thus, diploid *T. petrosa* has the typical embryo sac structure of a sexually reproducing grass.

For the tetraploid strain, 116 ovules at different stages of development were analysed. Approximately 40% of the observed ovules showed peculiarities at megasporogenesis and megagametogenesis, which were similar to those described for the diploid strain (Fig. 1*a*). However, aposporous embryo-sac development was observed in place of, or in addition to, meiotic embryo sac formation in most ovules. The process was characterised by the following steps: by the time the megaspore mother cell underwent meiosis, one or more surrounding nucellar cells enlarged, acquired dense cytoplasm, and showed a large and darkly stained

Table 1. Meiotic chromosome configurations at diakinesis and Metaphase I in pollen mother cells (PMCs) of *Thrasya petrosa*

Accession	2*n*	No. of PMCs	Average per PMC (range in parentheses)			
			I	II	III	IV
K2418	20	47	–	10	–	–
K1953	40	64	1.7 (0–5)	13.7 (8–20)	0.69 (0–3)	2.19 (0–5)

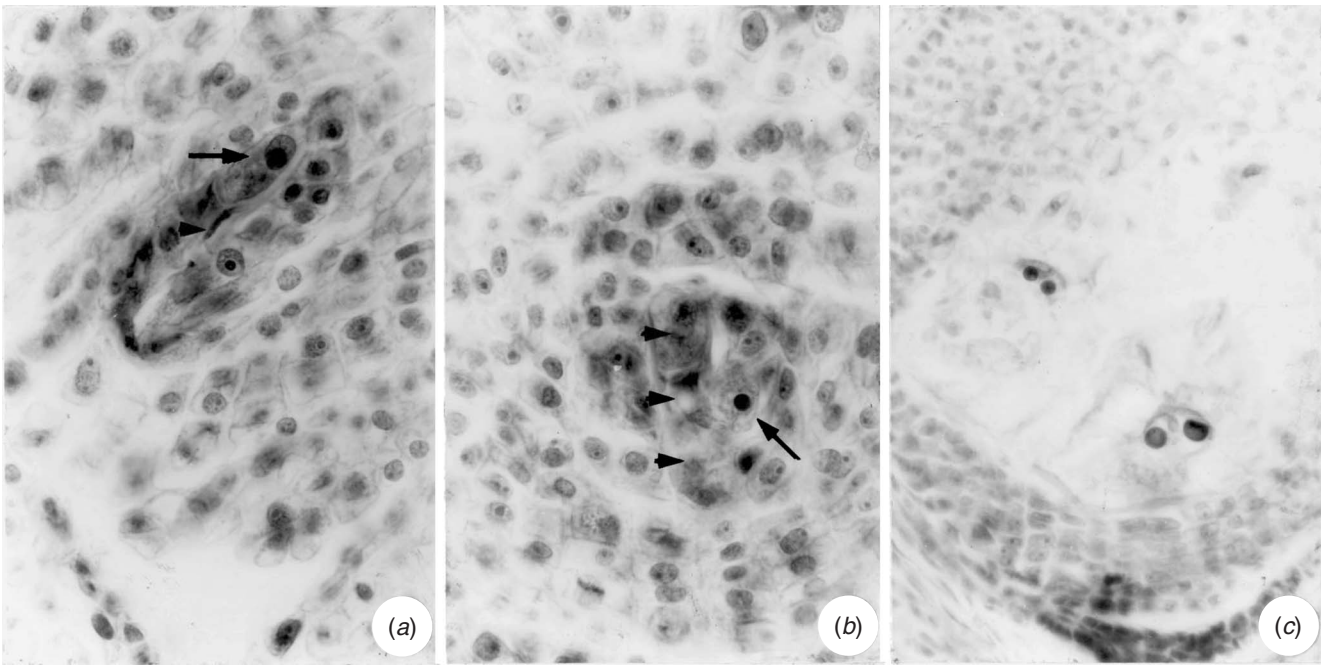

Fig. 1. Megasporogenesis and mature aposporous embryo sacs in ovules of tetraploid *Thrasya petrosa*. (*a*) The final stage of megasporogenesis with the functional megaspore (arrow) in the chalazal position and vestiges of the two degenerated megaspores towards the micropile (arrow head). (*b*) All three megaspores (arrow heads) at the beginning of the deterioration process revealed by the vanishing of nucleolus and nucleus, plus one aposporous embryo sac initial with darkly stained nucleolus (arrow). (*c*) Mature ovule with two aposporous embryo sacs. Each embryo sac shows two polar nuclei. The egg cells of these sacs were in adjacent sections of the ovule (not shown). Magnification: (*a*) and (*b*) ×645; (*c*) ×330.

nucleolus easy to distinguish from the nucleoli of normal nucellar cells. In some cases, these cytologically unreduced cells, initials of the aposporous embryo sacs, shared the central part of the ovule with the functional megaspore. However, in most cases, all meiotic products (the three megaspores) degenerated whereas aposporous initials were evident (Fig. 1*b*). One or more apospory initials underwent a first mitotic division of the nucleus to form bipolar developing embryo sacs. Few additional mitotic divisions led to the formation of aposporous sacs bearing an egg cell, and a large and widely vacuolated cell with two polar nuclei (Fig. 1*c*). Depending on the number of mitotic divisions, the mature aposporous sacs showed different nuclear configurations. The most common configuration was 5-nucleated with the egg, two synergids and two polar nuclei in the largely vacuolated cell, or 3-nucleated with the egg and the two polar nuclei. However, sacs with only one synergid or with three polar nuclei were also observed. Aposporous sacs always lacked antipodals. In summary, 10% of the mature ovules had only a meiotic sac (pure sexual), 53% had exclusively aposporous multiple sacs (pure aposporous), 31% had 1–3 aposporous sacs in addition to the meiotic one (mixed embryo sacs in a single ovule), and 6% had no sac at all as a result of abortion of the sporogeneous tissue and lack of aposporous embryo-sac development (sterile ovule). The embryological analysis indicated that the 4*x* strain of *T. petrosa* reproduces mainly by means of apomixis, with a natural ability for facultative sexuality. Theoretically, the probability of any ovary developing an apomictic embryo in this tetraploid plant is 53–84%, whereas the chances of sexual reproduction by fertilisation of the reduced egg cell in a meiotic embryo sac varies from 10 to 41%. This is because in those ovules with mixed embryo sacs (31%), it is supposed that the embryo may arise either after fertilisation of a meiotic megagametophyte or after parthenogenesis of a non-reduced egg cell from an aposporous sac.

Fertility

Studies using fluorescence techniques showed that pollen grains germinated and pollen tubes penetrated their own stigma papillae a few minutes after landing on the stigma surface in both diploid and tetraploid plants. However, in the diploid, the pollen tubes grew into the stigma papillae, but failed to penetrate the style. Apparently, a self-incompatibility system affected the pollen-tube growth in the central axis of the stigma or in the boundary region between the stigma and the style. In the tetraploid strain, the pollen tubes penetrated the stigma papillae and grew

through the stigma, style and ovary, following pollen-grain germination. Several pollen-tube tips were observed entering the ovule through the micropyle approximately 2 h after pollination.

Seed set was investigated in diploid and tetraploid plants. No seed formation was observed in 20 mature self-pollinated inflorescences of the diploid plant (approximately 1500 spikelets). The lack of seed formation provided additional evidence of a self-incompatibility system acting at diploid level in *T. petrosa*. The single tetraploid plant grown in the field nursery indicated its ability for self-pollination and self-fertilisation by setting seed. Notwithstanding, the performance of seed production was low (13.4%), probably owing to the poor fitness of this plant to the environmental conditions, presumably different from its natural habitat in eastern Bolivia. However, the need for pollination from another genotype could not be discarded since only one genotype was available.

Results from these studies of embryology, pollination behaviour and seed set suggest that the diploid strain of *T. petrosa* has a breeding system characterised by sexual reproduction and outbreeding, whereas the tetraploid strain showed apomictic reproduction with facultative sexuality.

Progeny test

The low seed production of the tetraploid plant and the poor seed germination meant that only 10 seedlings were obtained for the progeny test. Seedlings were potted and maintained in a glasshouse. Leaves from the mother plant and from each offspring were collected for DNA extraction. A total of 40 RAPD primers were tested. Eleven were discarded because of poor amplification. DNA fragment data from the remaining 29 primers were used to estimate the genetic relationship between the tetraploid mother plant (K1953) and each individual of the offspring. The 29 selected primers amplified many genomic regions, generating a total of 150 bands. All of these bands were shared by the mother plant and each of its progeny. An example of uniform bands obtained with three different primers is shown in Fig. 2. These results indicate that the 10-plant progeny was generated entirely by means of apomixis despite the observation that 10% of the ovules had meiotic sacs and 31% had mixed embryo sacs. However, the small number of individuals of this progeny does not allow rejection of the possibility of occasional sexual reproduction in tetraploid *T. petrosa*, as could be inferred from embryological data. Notwithstanding, the progeny test suggests that the most common pathway for reproduction in the tetraploid strain of this species was apospory. The genetic uniformity of the progeny could also be expected if the plants were derived from selfing of a largely inbred plant. However, the development of aposporous embryo sacs in 84% of the analysed ovules left little doubt about the apomictic origin of this progeny.

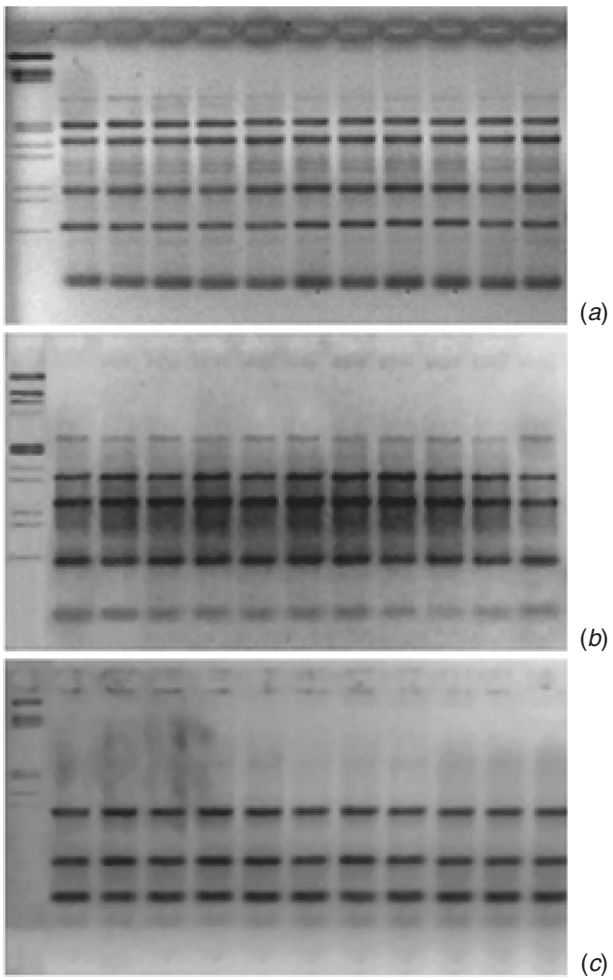

Fig. 2. Genetic fingerprints of *Thrasya petrosa* K1953 and its progeny obtained by self-pollination. Amplification by PCR and electrophoretic separation on a 2% agarose gel of genomic DNA from the mother plant K1953 and 10 progeny obtained by self-pollination. Amplification pattern of (*a*) BCU 405 primer, (*b*) BCU 449 and (*c*) BCU 497. Band patterns of the three primers were identical between the mother plant K1953 and its progeny. Lane 1 = molecular weight marker (Lambda *Hind*III from Promega), lane 2 = K1953, lane 3–12 = progeny 1–10.

Discussion

Multivalent chromosome associations that involved up to 50% of the chromosomes at meiosis suggest an autoploid origin for the tetraploid strain of *T. petrosa*. The natural existence of diploid plants of the same species and the close morphological similarities among 2*x* and 4*x* strains strengthen the hypothesis of autoploidy.

This is the first report of the existence of apomixis in the genus *Thrasya*. Thus, the information is enlarging the list of tropical and subtropical grass genera in which apomixis has decisive biological and evolutionary significance. Recent phylogenetic analyses using morphological studies

(Aliscioni 2002) as well as molecular data (Giussani *et al*. 2001) have shown that the genus *Thrasya* would fall into the paraphyletic genus *Paspalum*. Notwithstanding, these authors maintain *Paspalum* and *Thrasya* as different genera. *T. petrosa* shows the genetic system observed for a large number of *Paspalum* species. The system usually involves co-specific sexual diploid and apomictic tetraploid cytotypes. The diploid representatives are outbreeders due to self-incompatibility, whereas the apomictic tetraploids are pseudogamous and self-compatible (Norrmann *et al*. 1989; Quarin 1992). Our results indicate that *T. petrosa* shares these cytological and reproductive characteristics with *Paspalum*, a genus in which autoploidy has been suggested as the most likely origin for apomictic tetraploid species that have sexual diploid counterparts (Quarin 1992; Pupilli *et al*. 1997; Quarin *et al*. 1998). However, this genetic system is not restricted to *Paspalum* and *Thrasya*. Co-specific sexual diploids and apomictic polyploids are widespread in tropical *Panicoid* grass genera, although the system may show some particular variants. We observed in *T. petrosa* a genetic system very similar to that of several *Paspalum* species, and, therefore, our results do not contradict the taxonomic criterion that favours the inclusion of the species of *Thrasya* in the genus *Paspalum*.

The scarcity of our material and the difficulty in acquiring additional plant material from the wild or from other South American institutions made it difficult to assess how widespread apomixis is in the genus or even in the species. The occurrence of apospory in the tetraploid accession of *T. petrosa* is a very strong indication that apomixis is the common reproductive mode for this species at this ploidy level. In warm-season grasses, whenever apomixis was reported for some individual or some accession, it was generally assumed as a universal character for the species at that ploidy level. In other words, apomixis is usually a common reproductive character for all individuals of a given grass species at the same ploidy level, although the expression of the trait may vary from obligate to different degrees of facultative behaviour. For example, pentaploid *Paspalum dilatatum* Poir. has been reiteratively mentioned as apomictic (Hayman 1956; Bashaw and Holt 1958; Bashaw *et al*. 1970) but 100% sexual pentaploid individuals have never been reported for this species. A similar situation can be mentioned for tetraploid *Paspalum notatum* Flügge, tetraploid *Panicum maximum* Jacq. and many other polyploid races of apomictic warm-season grasses, for which only apomictic representatives are known. A rare exception is *Cenchrus ciliaris* L., for which Bashaw (1962) discovered a completely sexual plant among an apomictic tetraploid population. The opposite, a rare apomictic individual among a sexually reproducing grass species, has never been reported according to our knowledge. Thus, we can assume that the aposporous process that we observed in a limited tetraploid material of *T. petrosa* is a strong indication that apomixis is the common reproductive mode for tetraploid races of this species, whereas co-specific diploid races most likely reproduce sexually according to our results in a single diploid plant.

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References

Aliscioni SS (2002) Phylogeny of the genus *Paspalum* (Poaceae: Panicoideae: Paniceae). *Annals of the Missouri Botanical Garden* **89**, 504–523.

Bashaw EC, Holt EC (1958) Megasporogenesis, embryo sac development and embryogenesis in dallisgrass, *Paspalum dilatatum* Poir. *Agronomy Journal* **50**, 753–756.

Bashaw EC (1962) Apomixis and sexuality in buffelgrass. *Crop Science* **2**, 412–415.

Bashaw EC, Hovin AW, Holt EC (1970) Apomixis, its evolutionary significance and utilization in plant breeding. In 'Proceedings of the 11th international grassland congress'. (Ed. MJT Norman) pp. 245–248. (University of Queensland Press: Brisbane)

Burman AG (1985) Revision of the genus *Thrasya*. *Acta Botanica Venezuelica* **14**, 7–93.

Davidse G, Pohl RW (1974) Chromosome number, meiotic behavior, and notes on tropical American grasses (Gramineae). *Canadian Journal of Botany* **52**, 317–328.

Forbes I, Burton GW (1961) Cytology of diploids, natural and induced tetraploids, and intraspecies hybrids of bahiagrass, *Paspalum notatum* Flügge. *Crop Science* **1**, 402–406.

Giussani LM, Cota-Sánchez JH, Zuloaga FO, Kellog E (2001) A molecular phylogeny of the grass subfamily Panicoideae (Poaceae) shows multiple origins of C_4 photosynthesis. *American Journal of Botany* **88**, 1993–2012.

Hayman DL (1956) Cytological evidence of apomixis in Australian *Paspalum dilatatum*. *Journal of the Australian Institute of Agricultural Science* **22**, 292–293.

Martínez EJ, Hopp HE, Stein J, Ortiz JPA, Quarin CL (2003) Genetic characterization of apospory in tetraploid *Paspalum notatum* based on the identification of linked molecular markers. *Molecular Breeding* **12**, 319–327.
doi: 10.1023/B:MOLB.0000006868.12568.32

Nicora E, Rúgolo de Agrasar Z (1987) 'Los géneros de Gramíneas de América Austral.' (Editorial Hemisferio Sur: Buenos Aires)

Norrmann GA, Quarin CL, Burson BL (1989) Cytogenetics and reproductive behaviour of different chromosome races in six *Paspalum* species. *Journal of Heredity* **80**, 24–28.

Norrmann GA, Quarin CL, Killeen TJ (1994) Chromosome numbers in Bolivian grasses (Gramineae). *Annals of the Missouri Botanical Garden* **81**, 768–774.

Pohl RW, Davidse G (1971) Chromosome numbers of Costa Rican grasses. *Brittonia* **23**, 293–324.

Pupilli F, Cáceres ME, Quarin CL, Arcioni S (1997) Segregation analysis of RFLP markers reveals a tetrasomic inheritance in apomictic *Paspalum simplex*. *Genome* **40**, 822–828.

Quarin CL (1992) The nature of apomixis and its origin in Panicoids grasses. *Apomixis Newsletter* **1**, 8–15.

Quarin CL, Burson BL, Burton GW (1984) Cytology of intra and interspecific hybrids between two cytotypes of *Paspalum notatum* and *P. cromyorrhizon*. *Botanical Gazette* **145**, 420–426. doi: 10.1086/337474

Quarin CL, Norrmann GA, Espinoza F (1998) Evidence for autoploidy in apomictic *Paspalum rufum*. *Hereditas* **129**, 119–124. doi: 10.1111/j.1601-5223.1998.00119.x

Stein J, Quarin CL, Martínez EJ, Pessino SC, Ortiz JPA (2004) Tetraploid races of *Paspalum notatum* show polysomic inheritance and preferential chromosome pairing around the apospory-controlling locus. *Theoretical and Applied Genetics* **109**, 186–191. doi: 10.1007/s00122-004-1614-z

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