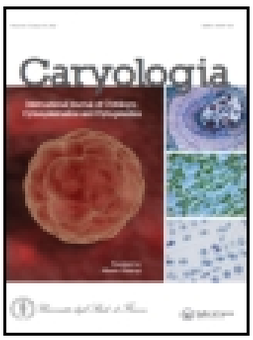

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Cytogenetics, genome size and anther anatomy in *Bulbostylis* (Cyperaceae)

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ABSTRACT

Cyperaceae display diverse and unusual cytogenetical characteristics, but are still sparsely documented and poorly understood in this respect. Six new chromosome counts are presented for *Bulbostylis*. Absolute DNA content was close to 1 pg in all species and was independent from chromosome number. Despite variations in chromosome number from $2n = 30$ to $2n = 102$, DNA content only varied from 0.932 pg to 1.198 pg. The studies presented in this work showed that species possess holokinetic chromosomes. The size of stomata and pollen grains did not vary. These results would confirm that the chromosome evolution of these species was via agmatoploidy and symploidy rather than by polyploidy. Abnormalities during meiotic spindle formation in microsporogenesis followed by simultaneous cytokinesis resulted in four microspores, of which only one was functional, resulting in a pseudomonad maturing into pollen grains with the usual vegetative and generative nuclei in addition to three degenerated microspore nuclei.

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KEYWORDS
Bulbostylis; holokinetic chromosomes; agmatoploidy; symploidy; pseudomonads

Introduction

Family Cyperaceae has a cosmopolitan distribution with a significant concentration of genera in the tropics. It includes about 5500 species in 109 genera (Govaerts et al. 2007), 14 tribes and four subfamilies (Goetghebeur 1998). Most species occur in wet habitats or submerged fields. Cyperaceae is a well-defined monophyletic group (Muasya et al. 1998, 2000) included in Poales (Angiosperm Phylogeny Group A. P. G. III, 2009) and sister to Juncaceae as supported by morphological and molecular studies (Simpson 1995; Bruhl 1995; Goetghebeur 1998; Muasya et al. 1998, 2000, 2009).

Cyperaceae display peculiar cytogenetic characteristics. They have holokinetic chromosomes (i.e. the kinetic activity extends along the entire chromosome), and pollen grains that develop from pseudomonads (Wahl 1940; Malheiros-Gardé and Gardé 1950, 1951; Hakansson 1954; Nordenskiöld 1962). Holokinetic chromosomes can be recognized by the variability of chromosomic fragments and the presence of a large kinetochore plate. Schrader (1935) was the first to observe the peculiar movement of chromosomes in the mitotic anaphase and to use the term "diffuse centromere".

Many organisms with holokinetic chromosomes have an uncommon meiotic behavior. The behavior is related to the orientation and migration of chromatids, which is characteristic of holokinetic chromosomes. The difference is observed at anaphase I: chromatids individualize, as if they were distinct units, and sister chromatids move apart from each other. In this phase, it is still possible to observe the remaining chiasmata. Because the separation occurs between sister chromatids, the division is equational (Nordenskiöld 1962). In contrast, in the anaphase I of monocentric chromosomes the separation is between homologous chromosomes. In Cyperaceae, holokinetic behavior has been studied only in *Carex* (Wahl 1940) and *Eleocharis palustris* (Hakansson 1954). In the sister family Juncaceae, it is known only from *Luzula purpurea* (Nordenskiöld 1962).

The absence of a single primary constriction and thus of a localized centromere has been termed in various ways, including "holocentric chromosomes", "with diffuse centromere or kinetochore" (Schrader 1935), or "non-localized centromere". However, the term "holocentric chromosomes" is the most appropriate (Bauer 1952; Sybenga 1972), because it implies that the kinetic activity is distributed along the entire length of the chromosome (=holokinetic) and not limited to a discrete region, as it is in monocentric (=monokinetic) chromosomes (Papeschi 1992). Therefore, the term "holokinetic chromosomes" reflects both the morphology and behavior of chromosomes (Mola and Papeschi 2006).

Karyotypic evolution in Cyperaceae is not only by polyploidy, but also by agmatoploidy (i.e. fragmentation of chromosomes) and symploidy (i.e. fusion of chromosomes). The term agmatoploidy was first used in the genus *Luzula* by Malheiros-Gardé and Gardé (1950).

The occurrence of fragmentations (agmatoploidy) and fusions (symploidy) of chromosomes in Cyperaceae makes it difficult to determine the basic chromosome

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number. However, the existence of several genera and species with chromosome numbers that are multiples of five suggests that the ancestral basic number in the family is *x*=5, while other basic numbers, such as *x*=2, 4, 6, and 12 are possible (Luceño et al. 1998a).

Another poorly studied aspect of Cyperaceae cytology is DNA content. DNA content has been measured only in a few species (Bennet and Leitch 1995): *Cyperus haspan*, *C. textiles*, *Eleocharis acicularis*, *Eriophorum vaginatum*, *Schoenoplectus lacustris* (=*Scirpus lacustris*) and *Schoenoplectus tabernaemontani* (=*Scirpus tabernaemontani*).

In this study, we focus on the genus *Bulbostylis* of tribe Abildgaardieae. Kral (1971) focused on the cytology at the tribe level and is the only published study including American *Bulbostylis*. Since he found that the basic number is $x = 5$, he concludes that polyploidy represents the evolutionary mechanism explaining the cytological diversity of the tribe and considers *Fimbristylis spathacea* an exception derived from another basic number, $x = 6, 8$. Despite his study, cytogenetic studies in *Bulbostylis* remain scarce (see Roalson 2008), and chromosome counts are known from only 16 of its 150 species (10.6% of the genus; see literature survey in Table 1). To fill this gap, we studied the cytology, microsporogenesis, and microgametogenesis of seven southern South American *Bulbostylis* species. In particular, we determined the mitotic chromosome number, the genome size, pollen grain and stomata size (to identify any correlation with chromosome numbers), and estimated pollen viability. Finally, we also described anther wall and pollen formation.

Material and methods

Material studied

Bulbostylis communis M.G. López & D. Simpson var. *communis* Argentina, Corrientes, Dpto. Monte Caseros, desembocadura del río Miriñay y río Uruguay; Playa arenosa. 12 March 2009. López 398 (CTES). Dpto. Capital, Laguna Soto, lomada arenosa. 16 July 1991. M. G. López 57 (CTES).

B. hirtella (Schrad.) Nees ex Urb. Argentina, Corrientes, Dpto. Monte Caseros, desembocadura del río Miriñay y río Uruguay. Barranca arenosa. 12 October 2008. López 391 (CTES).

B. juncoides (Vahl) Kük. ex Herter var. *juncoides*. Argentina, Córdoba, Dpto. San Alberto, Pampa de Achala; borde de Arroyo. 14 January 2009. López 388 (CTES).

B. rugosa M. G. López. Argentina, Corrientes, Dpto. Empedrado, frente al camping Municipal; barranca arenosa erosionada. 12 October 2009. López 389 (CTES).

B. sphaerocephala (Boeckeler) Lindm. var. *sphaerocephala*. Argentina, Misiones, Dpto. San Ignacio, Teyú Cuaré. 12 February 2009. López 394 (CTES).

B. sphaerolepis (Boeckeler) Beetle. Argentina, Corrientes, Dpto. Empedrado, frente al camping Municipal; barranca. 14 March 2006. López 378 (CTES).

B. subtilis M. G. López. Argentina, Misiones, Dpto. Eldorado, ruta provincial 17, paraje Cerro 60, cantera abandonada; entre piedras. 12 March 2009. López 393 (CTES).

Methods

The ploidy level of the seven studied species (see names and accession information in material studied) was determined by counting chromosomes (eight cells per species) in meristematic cells of root tips. Roots were pretreated in a saturated solution of α-bromonaphthalene for 2 h, hydrolyzed in HCl (1 N) for 10 min at 60 °C, and stained with Schiff's reagent. Finally, the root tips were smashed on a glass slide in a drop of 2% acetic orcein and analyzed with light microscopy.

For measurements of genome size, small leaf samples of similar size were taken from *Bulbostylis* plants and the standard *Paspalum dilatatum* Poir. ssp. *flavescens* Roseng. (Q 3952), and then placed together in a Petri dish (96 mm) with 0.5 ml nuclear extraction buffer from

Table 1. Chromosome numbers in *Bulbostylis* from previously published studies.

Species	2*n*	Source
Bulbostylis barbata (Rottb.) C.B. Clarke	10	Tanaka (1941) Nijalingappa (1972, 1975), Mehra and Sachdeva (1975)
	5 II	Kral (1971), Rath and Patnaik (1975, 1978), Bir et al. (1986, 1988, 1990, 1992)
	34 II	Bir et al. (1993)
B. capillaris (L.) Kunth ex C.B. Clarke	36 II	Sharma (1962)
B. ciliatifolia (Elliott) Fernald	30 II	Kral (1971)
B. cioniana (Savi) Lye	10	Elena Rossello and Gallego (1984)
B. densa (Wall.) Hand.-Mazz.	32 II	Ramachandran and Jeeja (1986)
B. densa ssp. *afromontana* (Lye) R.W. Haines	84	Tanaka (1937)
B. funckii (Steud.) C.B. Clarke	10 II	Kral (1971)
B. hirta (Thunb. ex Hoffm.) Svenson	15 II	Kral (1971)
B. hispidula (Vahl) R.W. Haines	5 II	Kral (1971)
B. junciformis C.B. Clarke	30 II	Kral (1971)
B. juncoides (Vhal) Kük. ex Osten	60 II	Kral (1971)
B. puberula C.B. Clarke	20	Ramachandran and Jeeja (1986)
B. stenophylla (Elliott) C.B. Clarke	15 II	Kral (1971)
B. trilobata Kral	25 II	Kral (1971)
B. vestita C.B. Clarke	30 II	Kral (1971)
B. warei C.B. Clarke	15 II	Kral (1971)

the kit Partec CyStain PI Absolute P (05-5022). Samples were ground with a razor blade. Later, they were filtered using a membrane, and the liquid was collected into 5 ml tubes including 1.5 ml of staining buffer of the kit CyStain PI absolute P (Partec). The tubes were incubated in the dark for 30 min and then analyzed using a flow cytometer PA II (Partec, Münster, Germany). The nuclear genome size of each sample was calculated using the formula: Sample 2C DNA content = [(sample G1 peak mean)/(standard G1 peak mean)] × standard 2C DNA content (pg DNA).

For estimates of pollen viability, pollen grains were stained with acetocarmine glycerol jelly, and 50 stainable pollen grains (i.e. those becoming intensively red colored) were measured and photographed using a 40× Zeiss-Axioplan microscope (Oberkochen, Germany) with a Canon PC1200 digital camera (Tokyo, Japan).

For stomata measurements, the technique of controlled maceration with nitric acid was used (Sass 1951). Samples of leaves were taken from herbarium specimens, placed in 40% nitric acid, and brought to boiling temperature (using a water bath). When the first boiling bubbles were observed, leaf samples were submerged in a separate container with water to extract the epidermis. In some cases it was necessary to use 50% sodium hypochlorite for a few seconds, while in other cases the epidermis was directly stained using safranin and then mounted in glycerinated water. Samples were photographed using a 40× Zeiss-Axioplan microscope with a Canon PC1200 digital camera. For each species, the size of 80–100 stomata was measured along the central leaf veins (stomata along the two marginal veins are smaller). InfoStat version 2014 was used for statistical analyses (Di Rienzo et al. 2014).

For anatomical studies, various developmental stages of floral buds of *Bulbostylis sphaerolepis* and *B. communis* were collected and fixed in FAA (alcohol, formaldehyde, acetic acid, 90:5:5, v/v). Samples were dehydrated following González and Cristóbal (1997) and subsequently infiltrated in paraffin (Johansen 1940). Transversal serial sections were cut at 10 µm with a Microm rotary microtome (Walldorf, Germany). Sections were stained with a safranin–astrablue combination (Luque et al. 1996), and then examined and photographed using a Leica DM LB2 light microscope (Bensheim, Germany).

For analyses of meiosis 30–50 spikes per accession were fixed in 5:1 absolute ethanol and lactic acid (Fernández 1973) at 4 °C, and then stored in 70% ethanol at the same temperature. Acetocarmine staining was used, and samples were observed using a light microscope, and photographs were taken using a Zeiss-Axioplan microscope with a Canon PC1200 digital camera.

Results

Mitosis, genome size, stomata size and pollen viability

Our analyses of mitosis revealed high variability in chromosome numbers (Table 2; Figures 1 and 2): *Bulbostylis communis* $2n = 20x = 102$ (Figure 1(C)), *B. hirtella* $2n = 20x = 103$ (Figure 1(F)), *B. juncoides* $2n = 96$ (Figure 1(I)), *B.* rugosa $2n = 48$ (Figure 1(L)), *Bulbostylis sphaerocephala* $2n = 6x = 30$ (Figure 2(C)), *B. sphaerolepis* $2n = 86$ (Figure 2(F)), *B. subtilis* $2n = 20x = 100$ (Figure 2(I)). Values of total DNA content ranged between 0.932 and 1.198 pg (Figure 3). Pollen grain size showed significant differences ($p>0.05$) between species (Table 2, and Figures 1(B), 1(E), 1(H), 1(K), 2(B), 2(E), and 2(H)).

Stomata size (Table 2) was similar in *B. sphaerocephala* ($2n = 30$, Figure 2(A)) and *B. subtilis* ($2n = 100$, Figure 2(G)), but showed significant differences with *B. rugosa* ($2n = 48$, Figure 1(J)) and *B. hirtella* ($2n = 103$, Figure 1(D)), whereas *B. communis* ($2n = 102$, Figure 1(A)) and *B. juncoides* ($2n = 96$, Figure 1(G)) were similar but significantly different to *B. sphaerolepis* ($2n = 86$, Figure 2(D)), which present larger stomata, with varying sizes within the same leaf. Pollen stainability ranged between 25% and 70% in all species but *B. communis*, where it was 96%.

Anatomy of the anther

Transversal sections show that mature anthers have four pollen sacs, and their wall consists of four layers: epidermis, endothecium, middle layer, and tapetum. At initial developmental stages, three layers can be seen: an external layer that corresponds to the epidermal layer, a subepidermal layer that will form the endothecium, and an internal layer that will divide periclinally to form the only middle layer and the tapetum (Figure

Table 2. Chromosome number, total DNA content 2C values in picograms (pg), pollen size, stomata size, and pollen viability in *Bulbostylis*.

Bulbostylis species	2*n*	2C (pg) SE	Pollen size in µm SE	Stomata size in µm SE	Pollen viability (%)
B. communis	102	0.932 ± 0.008	28.94 ± 3.94$^{a, b}$	43.35 ± 4.60^{d}	96.26
B. hirtella	103	1.050 ± 0.040	30.56 ± 3.06^{c}	33.50 ± 2.50^{b}	52.75
B. juncoides	96	1.098 ± 0.030	32.93 ± 2.93^{d}	43.29 ± 4.54^{d}	25.32
B. rugosa	48	1.076 ± 0.048	27.95 ± 2.95$^{a, b}$	33.49 ± 2.50^{b}	77.98
B. sphaerocephala	30	0.982 ± 0.062	29.94 ± 2.44$^{b, c}$	29.62 ± 1.62^{a}	59.76
B. sphaerolepis	86	1.198 ± 0.007	33.03 ± 3.03^{d}	41.33 ± 5.68^{c}	76.04
B. subtilis	100	1.012 ± 0.022	28.43 ± 3.43^{a}	29.40 ± 1.72^{a}	62.76

Abbreviation: SE, standard error. Means followed by the same letter are not significantly different. Tukey test ($p = 0.05$).

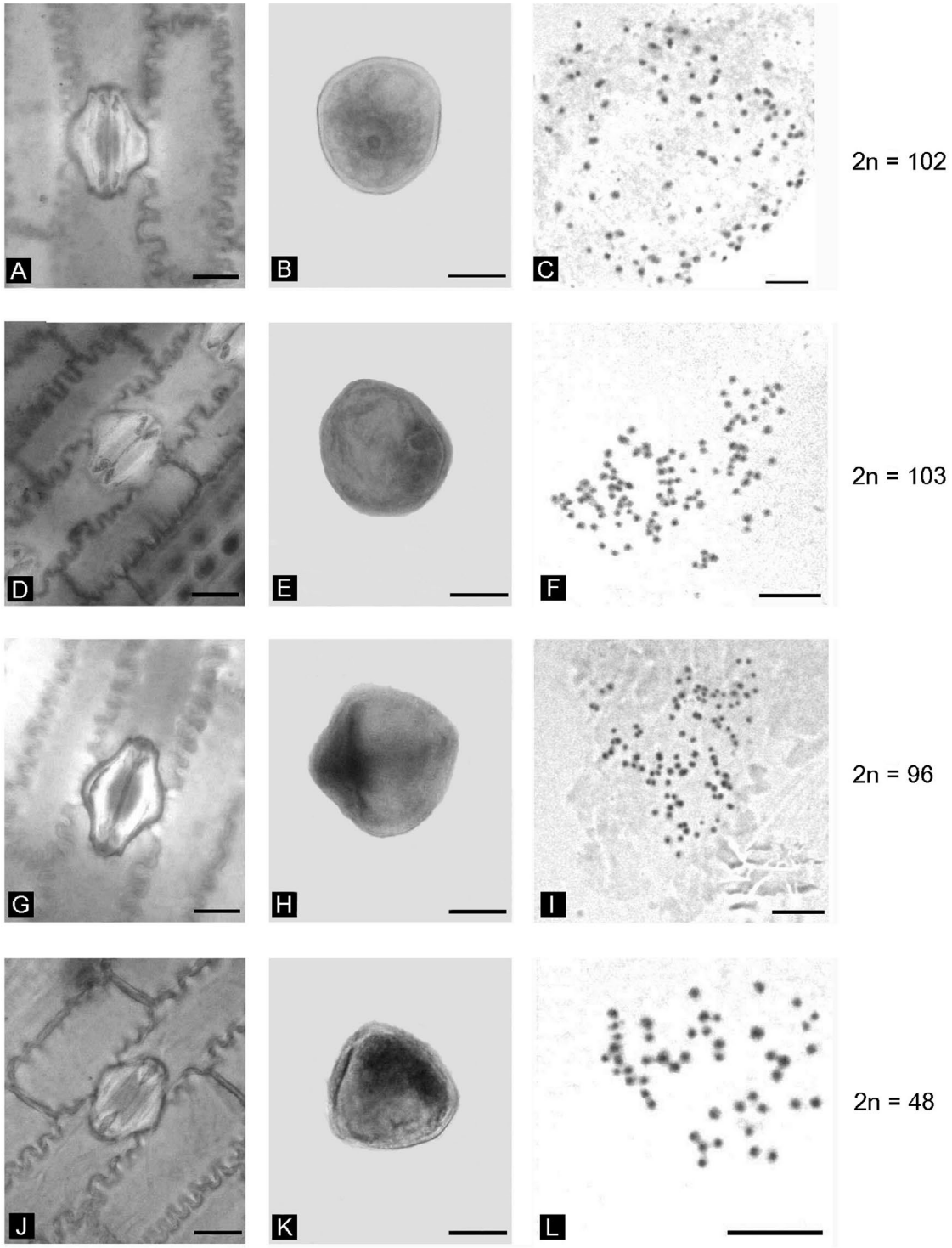

Figure 1. (A, D, G, J) Stomata superficial view (scale bar = 10 µm). (B, E, H, K) Pollen grains (scale bar = 10 µm). (C, F, I, L) Mitosis (scale bar = 5 µm). (A–C) *Bulbostylis communis* from López 398 (CTES). (D–F) *Bulbostylis hirtella*, from López 391 (CTES). (G–I) *Bulbostylis juncoides*, from López 388 (CTES). (J–L) *Bulbostylis rugosa*, from López 389 (CTES).

4(A) and 4(B)). The tapetum is uniseriate, uninuclear, and secretory (Figure 4(B) and 4(C)). During anther and tapetum maturation, the middle layer disappears (Figure 4(C–E)). The few and large pollen mother cells (hereafter PMC) arrange themselves in a peripheral position and in groups of three or four in each of the pollen sacs (Figure 4(A–C)). As expected, transversal sections indicate that anthers correspond to the monocot type of Kirpes et al. (1996).

Microsporogenesis and microgametogenesis

The first meiotic division results in two nuclei lacking a partition wall between them (Figure 5(A) and 5(B)). Figure 5(A) illustrates the large quantity of microtubules characteristic of holocentric chromosomes. The second division occurs shortly after the first, resulting in four nuclei (Figure 5(C–F)). The four nuclei are initially similar in size, but the external one (near the tapetum)

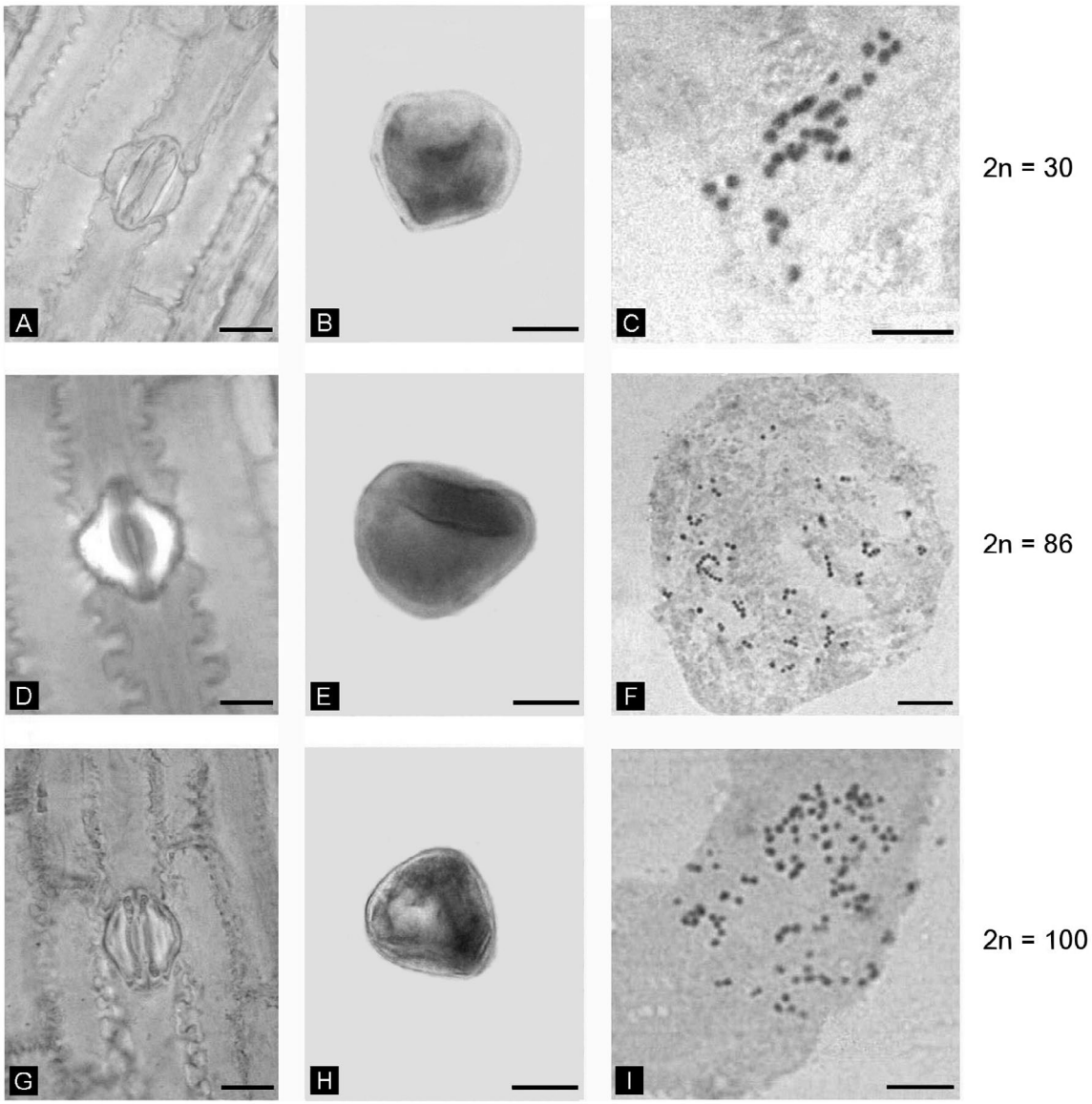

Figure 2. (A, D, G) Stomata superficial view (scale bar = 10 µm). (B, E, H) Pollen grains (scale bar = 10 µm). (E, F, I) Mitosis (scale bar = 5 µm). (A–C) *Bulbostylis sphaerocephala*, from López 394. (D–F) *Bulbostylis sphaerolepis*, from López 378 (CTES). (G–I) *Bulbostylis subtilis*, from López 393.

becomes larger and surrounded by a large cytoplasmic area and displaces the other three abortive nuclei towards the corner that is opposite to the locule center (Figure 5(G) and 5(H)). Nucleus migration of the tetrads is the first sign of the intracellular polarity and determines all following events.

The first mitotic division occurs simultaneously in the four nuclei (Figure 5(I)). The peripheral nuclei form micronuclei or, in some cases, an irregular division. The central nucleus divides into a generative nucleus and a vegetative nucleus (Figure 5(J–L)).

Discussion

Analysis of mitosis

We found a high variability of chromosome numbers in *Bulbostylis*; all have the basic number $x = 5$. Chromosomes numbers resulted from fragmentation in most cases, and from fusion in the remaining few cases. For example, the numbers of *B. communis*, $2n = 20x = 102$, and *B. hirtella* $2n = 20x = 103$, could have resulted from an initial $2n = 20x = 100$ followed by fragmentation. *Bulbostylis juncoides,* $2n = 96$, could have resulted from an initial $2n = 18x = 90$ followed by fragmentation, or from an initial $2n = 20x = 100$ followed by chromosome fusion. Interestingly, Kral (1971) studied meiosis in this taxon and discovered the presence of $n = 60$. In the case of *B. rugosa*, $2n = 48$ and *B. juncoides*, $2n = 96$, the basic number could be secondary as $x = 6$ as *Bulbostylis capillaris* (Sharma 1962). This species would be an octoploid resulting from a decaploid where chromosomes fused to form $2n = 48$. Finally, in *B. sphaerolepis*, its $2n = 86$ could have resulted from an initial $2n = 18x = 90$ followed by fusion or from $2n = 16x = 80$ followed by fragmentation.

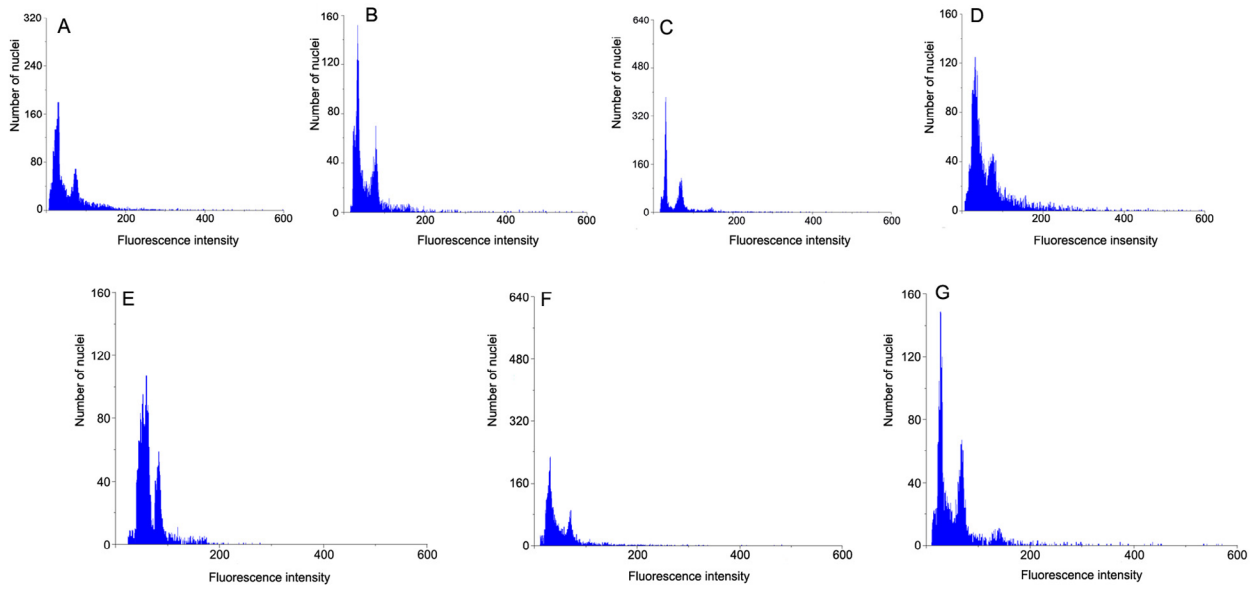

Figure 3. Histograms of flow cytometry analyses. (A) *Bulbostylis communis*; (B) *Bulbostylis hirtella*; (C) *Bulbostylis juncoides*; (D) *Bulbostylis rugosa*; (E) *Bulbostylis sphaerocephala*; (F) *Bulbostylis sphaerolepis*; (G) *Bulbostylis subtilis.* The standard *Paspalum dilatatum* Poir. ssp. *flavescens* Roseng. (Q 3952) was used for all analyses.

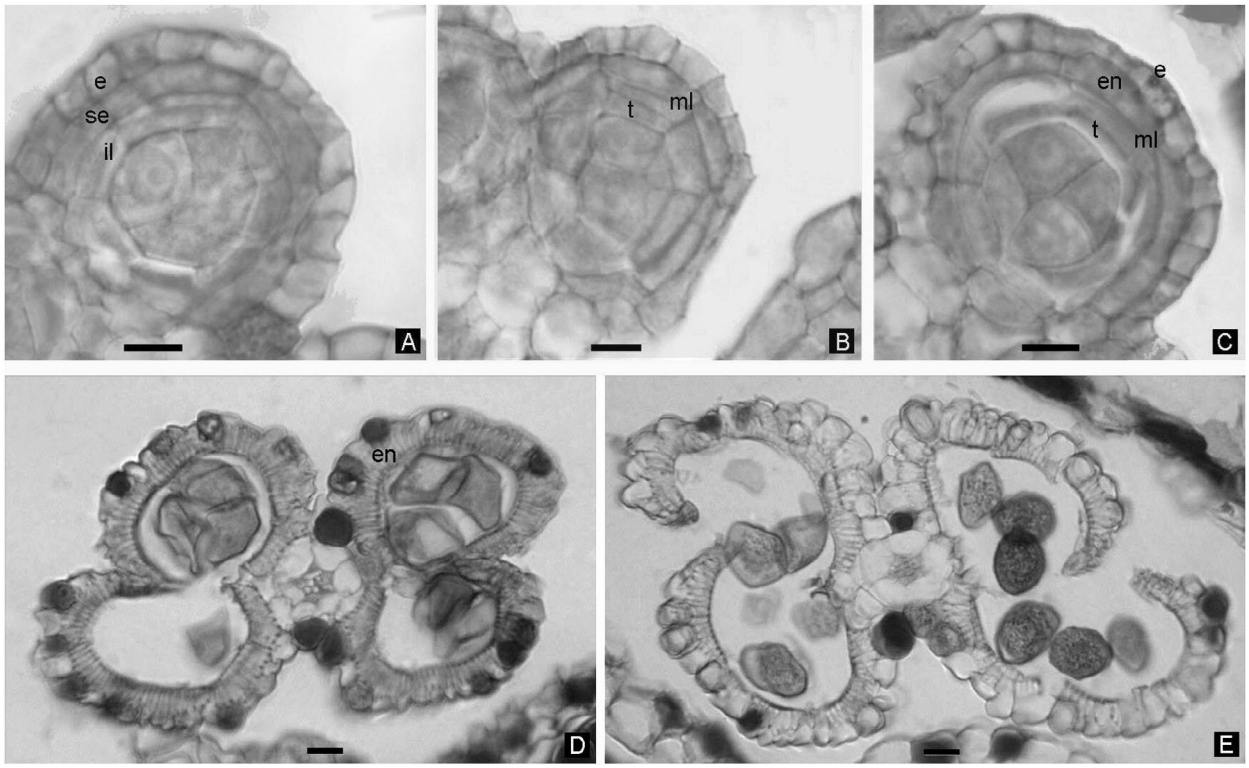

Figure 4. Structure of the anther wall of *Bulbostylis communis*. (A–C) Transversal section of a young anther, showing wall differentiation in one of the 4 locules. (D, E) Transversal section of a complete mature anther. (A) Pollen sac with three of the four walls: epidermal (e), subepidermal (se) and internal layer (il). (B) Periclinal division and internal layer originating the middle layer (ml) and the tapetum (t). (C) Mature anther with cells remaining from the middle layer (ml) and tapetum (t). (D) Microsporocyte: endothecium (en) and pollen grains. (E) Dehiscence. Voucher specimen used: M.G. López 57 (CTES). Scale bar = 25 µm.

Genome size

To know whether karyotypic evolution in organisms with holokinetic chromosomes occurred via polyploidy, agmatoploydy (fragmentation), or symploidy (fusion),their karyotype can be analyzed measuring the total length of chromosomes (Luceño, et al. 1998b; Dopchiz et al. 2000; Vanzela and Guerra 2000) and/or the DNA content, i.e. c-value (Mello-Sampayo 1961; Halkka 1964; Kuta et al. 2004). In all studied *Bulbostylis* species, we found a DNA content approximating 1 pg,

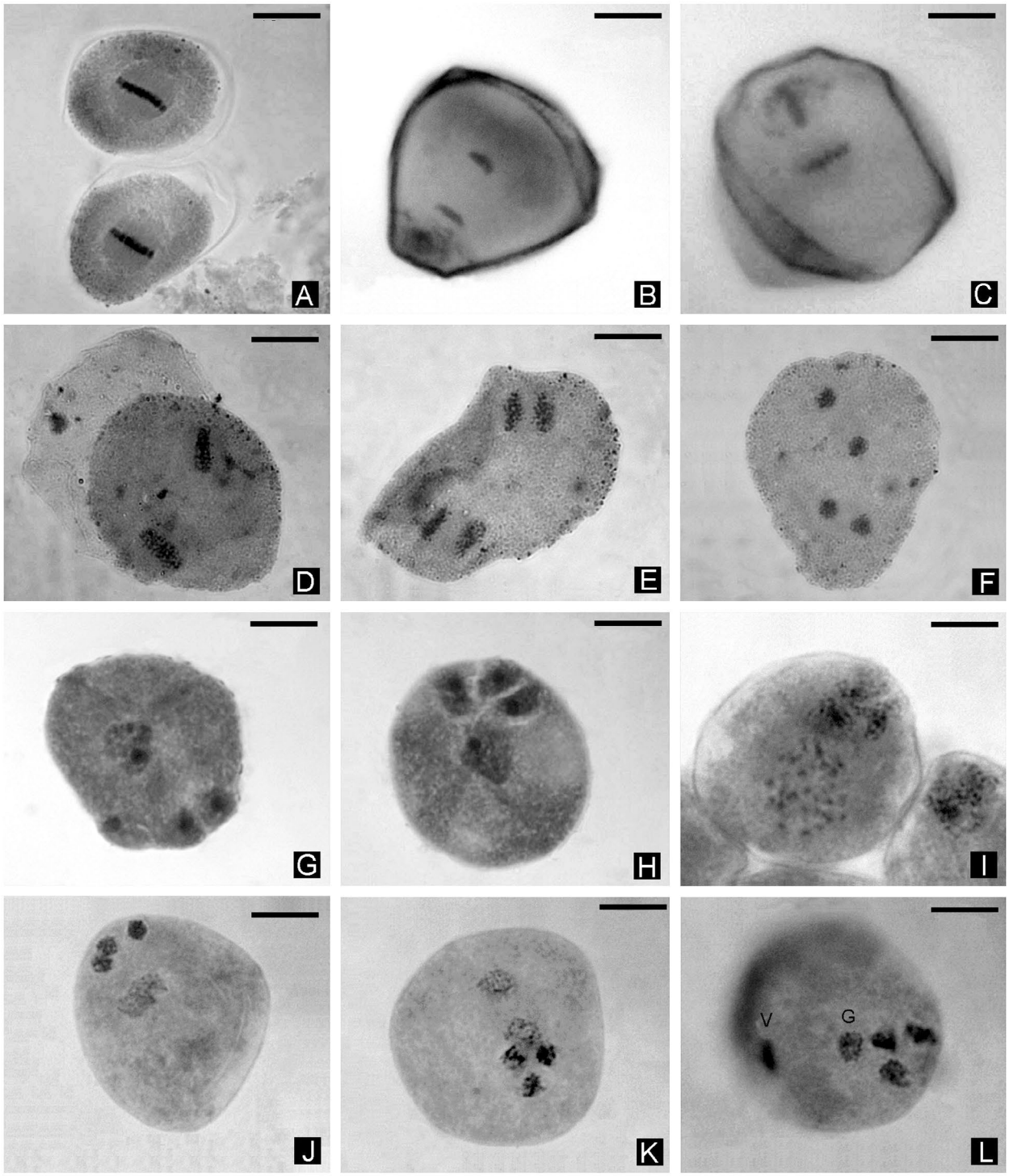

Figure 5. Meiosis in species of *Bulbostylis*. (A) Metaphase I. (B) Anaphase I. (C) Metaphase II. (D) Early anaphase II. (E) Anaphase II. (F) Telophase II. (G) Functional nucleus in the center of the cell. (H) Functional nucleus near the abortive cells and preparing for mitosis. (I) Mitotic prophase of the functional cell. (J, K) Mitotic telophase. (L) Generative (g) and vegetative (v) cells. Voucher specimens used: (A–C) and (K–L) *Bulbostylis communis* from M. G. López 50. (D–J) *B. rugosa* from M. G. López 389. Scale bars in A = 12 µm, in B–L = 7 µm.

independently of their chromosome number, suggesting that agmatoploidy and symploidy played an important role in the evolution of these species. Agmatoploidy and symploidy appear to have played similar roles also in other Cyperaceae genera, such as *Rhynchospora* (Luceño et al. 1998a, 1998b; Dopchiz et al. 2000; Vanzela and Guerra 2000), *Carex* (Luceño and Castroviejo 1991), and *Eleocharis* (Da Silva 2005).

Holokinetic chromosomes

Holokinetic chromosomes have been described in a few groups of plants and invertebrates. In plants, such chromosomes are so far known in the monocot families Cyperaceae and Juncaceae (Mello-Sampayo 1961; Greilhuber 1995; Luceño et al. 1998a; Vanzela and Guerra 2000; Kuta et al. 2004), in the genera

Chionographis (Melanthiaceae), *Drosera* (Droseraceae), *Myristica* (Myristicaceae) and *Cuscuta* subgen. *Cuscuta* (Convolvulaceae), and in a few species of families Zingiberaceae, Musaceae and Cannaceae (Mola and Papeschi 2006). In invertebrates, holokinetic chromosomes have been reported in the insect orders Homoptera, Hemiptera, Lepidoptera, and Heteroptera (Papeschi 1992) as well as in all nematodes (Mola and Papeschi 2006).

There are several hypotheses for the origin of holokinetic chromosomes in plants and animals (Mola and Papeschi 2006). One of them considers holokinetic chromosomes as more "primitive" than monocentric (monokinetic) chromosomes, which have a localized structure including the centromere. This hypothesis is based on the following aspects: (a) localized centromeres are specialized structures; (b) holokinetic chromosomes are generally found in taxa of plants and animals from older lineages; (c) fragmentations increase proportionally to the chromosome number, leading to a higher variability and a higher adaptation ability; and (d) fusions and translocations are less constrained in holocentric chromosomes than in monocentric chromosomes (see Schrader 1947; Castro 1950; Camara 1953; Vaarama 1954; Kiauta 1970). Another hypothesis is that the structure of holokinetic chromosomes is actually derived, because these chromosomes are found in phylogenetically distantly related groups. Greilhuber (1995) supported this hypothesis based on outgroup comparisons, considering that holokinetic chromosomes derived in angiosperms independently at least four times: in Junceceae–Cyperaceae, in *Chionographis* (Melanthiaceae), in *Cuscuta* subgenus *Cuscuta* (Convolvulaceae) and in *Myristica fragrans* (Myristicaceae). He also considered the presence of holokinetic chromosomes associated with meiotic inversion in the Cyperaceae–Juncaceae group as the only good synapomorphy for monocots. According to him, a larger expansion of the kinetic activity could have occurred incidentally through a transposable element with a centromeric sequence.

According to the hypothesis that considers holokinetic chromosomes as more "primitive" than the monocentric ones (Mola and Papeschi 2006), fragmentations increase the number of chromosomes, leading to a higher variability and, ultimately, to a superior adaptability. This seems to happen in the species of *Bulbostylis* studied here, since they are polymorphic and ecologically diverse, grow in diverse types of soil, and also have a long phenology, flowering and fruiting throughout the entire year, or most of it.

Microsporogenesis and microgametogenesis

We found no correlation between duplications in chromosome numbers and increase in pollen size and stomata size in *Bulbostylis*, demonstrating for the first time that the "gigas effect" (Stebbins 1971), commonly observed in polyploids, does not occur in agmatoploidy. Pollen viability was low; data on fertility did not correlate with chromosome numbers, but this could be due to the weak exine in Cyperaceae pollen, which makes them collapse more easily.

The end result of the meiotic division of the wedge-shaped PCM is a large functional microspore, located in the wider area towards the tapetum (abaxial), and three small abortive microspores, located in the narrower area towards the locule center (adaxial), undergoing a simultaneous cytokinesis. Such cytokinesis is reported to occur only in 20% of monocots (Furness and Rudall 1999). The behavior in the four nuclei is similar to the development of the female gametophyte (Brown and Lemmon 2000). In the post-meiotic developmental stages, they reflect an intra- and intercellular polarization. The four nuclei resulting from the meiosis segregate uniformly in the microsporocyte. The nuclei form a compact tetrad in the center, and then move together towards the pole in the center of the anther locule. The nucleus located towards the center of the microsporocyte is the functional one; it becomes larger and moves to a central position. The nucleus of the functional microspore migrates toward the proximal surface (near the abortive microspores) getting ready to undergo mitosis.

All microspores, one functional and three abortive, undergo mitosis, but mitosis of the abortive microspores stops at metaphase and the abortive microspores degenerate. The functional (generative) cell remains adjacent to the three abortive microspores, and the vegetative nucleus migrates towards the central part of the functional microspore. The relation between the cytoplasm volume of the viable nucleus and that of each of the abortive nuclei is 8:1 (Tanaka 1939). We show that pseudomonads are formed in *Bulbostylis*, resulting in one pollen grain for each mother cell.

In conclusion, the *Bulbostylis* species that we studied have holokinetic chromosomes and tend to maintain the basic number $x = 5$ of the Cyperaceae family. However, we report the secondary basic number $x = 6$. Our study demonstrates that agmatoploidy in particular, and, to a lesser extent, symploidy, played an important role in the karyotypic evolution of *Bulbostylis*. According with Kral (1971), the formation of pseudomonads in microsporogenesis has long been observed even in *Bulbostylis*. What is new in the present paper is therefore more sequential photographic documentation of the process and this should be more clearly pointed out.

Disclosure statement

No potential conflict of interest was reported by the authors.

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