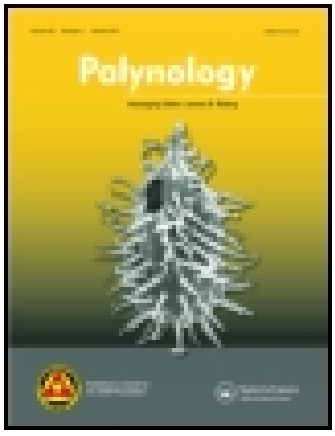
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Comparative pollen morphology and viability among endangered species of Butia (Arecaceae) and its implications for species delimitation and conservation

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Comparative pollen morphology and viability among endangered species of *Butia* (Arecaceae) and its implications for species delimitation and conservation

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The present study reports the results of pollen analyses on four species of *Butia* (Arecaceae), *Butia odorata*, *B. yatay*, *B. paraguayensis* and *B. lallemantii.* Pollen grains were described using light microscopy (LM) and scanning electron microscopy (SEM), and pollen viability was determined by the fluorochromatic reaction (FCR) test. All species of *Butia* studied produce monosulcate pollen grains with a tectate perforate exine. Additionally, *B. odorata* and *B. paraguayensis* produce two pollen grain types, mono- and also trichotomosulcate, which has been considered a derived state of the character. Regarding pollen grain size and shape, there are significant differences in long and short axis lengths, and their ratio. Despite serious regeneration problems which jeopardise population survival in the short-term future, results showed that pollen viability of all species of *Butia* analysed was high enough to ensure good pollination. Therefore, pollen viability is not the limiting factor for population continuity. This is the first study dealing both with pollen morphology and assessment of pollen viability with the aim of species delimitation within this genus. Additionally, this is the first study providing new information regarding the status of the current *Butia* populations of southern South America from a palynological point of view.

Keywords: *Butia*; Palmae; pollen differentiation; pollen viability; population continuity

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1. Introduction

The palm family (Areceaceae, Bercht & J. Presl 1832) comprises about 2640 species in about 230 genera widely distributed in the tropics and subtropics, while only a few species are found in warm-temperate regions (Dransfield et al. 2008). The genus *Butia* (Becc.) (tribe Cocoseae, subtribe Attaleineae) is distributed exclusively in South America, across Brazil, Paraguay, Argentina and Uruguay. Of all *Butia* species, four have their southern distribution limit in Uruguay: *Butia odorata* (Barb. Rodr.) Noblick, *B. yatay* (Mart.) Becc., *B. paraguayensis* (Barb. Rodr.) L.H. Bailey and *B. lallemantii* Deble & Marchiori (see Figure 1). *Butia odorata*, *B. lallemantii* and *B. paraguayensis* are threatened species in Uruguay and were recently defined as a priority for conservation (Marchesi et al. 2013). *Butia* species show disjunct distributions in Uruguay (Figure 1). *Butia paraguayensis* and *B. lallemantii* are restricted to small populations in the north, which consist of very few individuals, mostly adults and with varying degrees of damage by pests and diseases. On the other hand, *B. odorata* and *B. yatay* form larger populations with enormous economic and scenic value. All of the species included in this study are exposed to demographic threats such as aging populations and lack of recruitment. Cattle grazing and forestation have fragmented and reduced these natural populations, posing an additional threat which jeopardises their continuity in the short-term future (Brussa & Grela 2007). Nevertheless, genetic diversity within populations evaluated by neutral molecular markers (Inter-Simple Sequence Repeats - ISSR) is high (Gaiero et al. 2011). This would provide genetic potential for recovery if other steps were taken towards their demographic restoration (Gaiero et al. 2011).

In order to make management decisions towards species conservation, clear and uniform species identification is a top priority (Frankham et al. 2002). Taxonomy in the genus *Butia* is complex, and species delimitation remains unclear. Phylogenetic studies on subtribe Attaleineae based on the analysis of seven WRKY genes (Meerow et al. 2009) suggested that species-level divergences within *Butia* occurred in the last 10 million years, but no further internal resolution was

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Figure 1. Distribution of species of *Butia* within Uruguay. Adapted from Chebataroff (1974) and Brussa & Grela (2007).

found. Genetic studies in natural populations of *Butia* species show that even though the species delimitation employed so far seems to be the most appropriate, genetic proximity among *Butia* species does not allow the authors to consider them as clearly separate genetic groups (Gaiero et al. 2011). Regarding their morphology, *Butia* species form a highly diverse group, hence the taxonomic doubts surrounding the genus and its species delimitation (Marcato 2004). Additional highly informative morphological characters are needed to contribute to species delimitation.

Pollen morphology can be applied as a taxonomic character in Arecaceae. Most palm species produce pollen grains of the same type, simple tectate or semitectate, columellate, monosulcate (Mahabalé 1967; Harley 1990). Nevertheless, for a monocotyledonous family, Arecaceae has a remarkable range of variation in pollen aperture types, where trichotomosulcate, equatorial disulcate and porate pollen grains also occur, as well as different exine ornamentations such as scabrate, perforate, verrucate and reticulate (Sowunmi 1968, 1972; Thanikaimoni 1970; Harley 1990, 1996; Ferguson & Harley 1993; Harley & Baker 2001; Harley & Dransfield 2003; Machado Rodríguez 2003). However, species from a genus are usually similar in their aperture form and exine patterns, and they are mainly distinguished by their relative pollen grain size (Sowunmi 1972). Pollen grains from *Butia* and other palm genera have been analysed through light microscopy in Brazilian populations occurring in the state of Rio Grande do Sul (Bauermann et al. 2010). The authors found that the species of *Butia* studied (*B. odorata*, *B. eriospatha*, *B. yatay* and *B. paraguayensis*) displayed larger pollen grains (for long axis in polar view) than the rest of the species included in the study (from genera *Euterpe*, *Geonoma*, *Syagrus* and *Thritrinax*), and could be grouped according to this characteristic. However, exine ornamentations and aperture types did not allow discrimination within the genus *Butia* (Bauermann et al. 2010). There are no studies on fresh material of these species, since samples for that study were taken from herbarium specimens. Moreover, different populations could display different morphologies due to interpopulation variation, as found for other characters by Rivas & Barilani (2004).

Pollen grains are a major component of the reproductive biology of plant species. Reproductive success is greatly affected by pollen dispersal and effective pollination within and among populations. Together with seed germination and seedling survival, these are key elements in population continuity and influence connectivity in fragmented habitats (Aizen & Feinsinger 1994; Hamrick 2004). Pollination in *Butia* is likely to be entomophilous, although anemophilous pollination cannot be disregarded (Jones 1995). Animal-pollinated species are strongly negatively affected, in terms of effective pollination service and seed production, by habitat fragmentation (Aizen & Feinsinger 1994; Aguilar et al. 2008). Insufficient arrival of pollen grains on the stigma commonly compromises reproductive success in plant populations (Knight et al. 2005), and poor quality of pollen grains reduces seed production significantly (Aizen & Harder 2007; Harder & Aizen 2010). Low pollen viability impedes effective pollination, threatening the reproductive capacity of individuals and therefore population survival, especially in small and isolated populations. Pollen viability can be indirectly estimated by fluorochromatic reaction (FCR), an easy and quick method that shows high correlation with pollen grain germination and ability to deliver sperm cells to the embryo sac (Shivanna et al. 1991).

Butia species are facing demographic challenges, while their species delimitation and characterisation remain unsolved. Pollen grain characteristics are critical for effective reproductive functioning, as well as being useful species descriptors. Therefore, our objectives were to characterise pollen grains from natural populations of *Butia* species. Specifically, we compared morphological differences in pollen grains among *Butia* species using light microscopy (LM) and

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scanning electron microscopy (SEM). LM allows the identification and interpretation of the pollen spectrum of a particular sample, whereas SEM is mainly used for morphological comparisons and taxonomy, because of its increased resolution (Jones & Bryant 2007), and its role to illustrate exine sculpture and structure (Harley & Ferguson 1990). Additionally, we compared pollen viability among species in order to evaluate their effective pollination ability. Our results provide novel palynological data for *Butia* populations of southern South America, and useful information to assist species delimitation within the genus.

2. Materials and methods

Pollen morphology and viability of the four *Butia* species distributed in Uruguay (*B. odorata*, *B. yatay*, *B. paraguayensis* and *B. lallemantii*) were studied. Field work was carried out in the flowering seasons of 2008 and 2009 (November to February). Mature but closed inflorescences from at least two individuals of each population were collected and kept in flower vases in the laboratory. Specimens of each species were previously identified by Dr. Larry Noblick and deposited in the 'Bernardo Rosengurtt' Herbarium (MVFA), Facultad de Agronomía, Universidad de la República (Montevideo, Uruguay). Their herbarium identification as well as the geographical coordinates of collected samples are described in Table 1, together with a description of the populations in which they were found.

2.1. Light microscopy

Immediately after spathe opening, fresh pollen grain samples were collected from inflorescences and acetolysed following Erdtman (1960), with timing at 100°C restricted to 2 minutes. After acetolysis, pollen grain samples were mounted in glycerol jelly and examined using an Olympus System Microscope Model CX41. In order to minimise alteration of pollen grain size on the slide, 50 pollen grains per individual were measured on the same day they were mounted. Following specific

Table 1. Geographical coordinates of collected samples, 'Bernardo Rosengurtt' Herbarium (MVFA) exsiccate identification and collection site description.

Taxon/sample code	Location		MVFA exsiccate identification	Collection site description
	Latitude	Longitude		
B. odorata	S34°09′41.60″	W53°48′18.10″	L.R. Noblick 5466	Small population in a man-made park
BOPR1	S34°51′55.78″	W56°01′10.53″		Palm grove on grassland with cattle grazing
BOR9	S34°11′40.74″	W53°46′01.70″		
BO3	S34°11′23.66″	W53°46′50.89″		
B. yatay	S31°55′37.00′′	W57°51′17.50′′	L.R. Noblick 5460	Palm grove on grassland with cattle grazing and forested areas
BYQ2	S31°54′36.80′′	W57°39′32.40′′		Small population on grassland
BYQ3	S31°54′37.54″	W57°39′34.53″		
BYQ4	S31°54′38.28″	W57°39′36.51″		Palm grove on plateau of a hill; herbaceous strata composed of Poaceae and Asteraceae with cattle grazing
BYGUI	S32°20′39.37″	W57°09′28.04″		
B. paraguayensis	S31°32′04.60″	W55°37′56.00″	L.R. Noblick 5459	
BP4	S31°32′01.29″	*W55°37′58.47″		
BP5	S31°32′00.80″	*W55° 37′58.30″		
BP6	S31°32′01.07”	*W55°37′59.68″		
B. lallemantii	S30°59′51.30″	W55°39′40.80″	L.R. Noblick 5457	Small population on open fields and pine forested areas
BLC	S30°59′37.53″	W55°41′16.11″		Small population on sandstone slopes and ledges; gulch forest
BLPL49	S31°00′05.96″	W55°41′00.02″		
BLPL57	S30°59′56.90″	W55°40′30.97″		

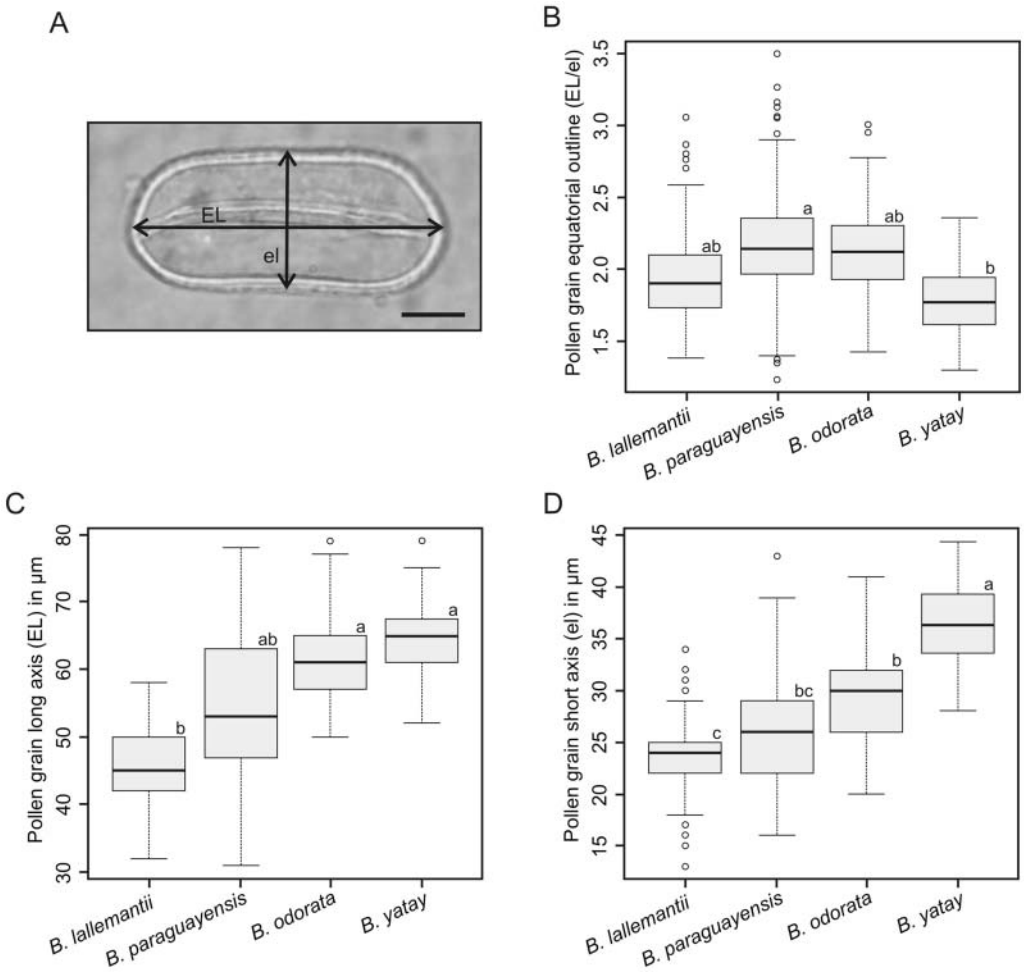


Figure 2. A. Light microscope micrograph of a monosulcate pollen grain in polar view with the measured variables. Scale bar: 10 μm. B–D. Variation of pollen grains in the four *Butia* species studied for: B. Equatorial outline expressed as the ratio of equatorial long axis (EL) to equatorial short axis (el); C. EL in μm; D. el in μm. The central horizontal line on box-plots represents the median length of the pollen of each species. The box comprises the upper and the lower quartile (i.e., 25th and 75th quartile). The whiskers represent the median plus 1.5 the interquartile range. Extreme values (outliers) are represented as dots outside the whiskers. The same letters indicate no statistical differences between species ($\alpha = 0.05$).

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nomenclature for monocots as described by Ferguson & Harley (1993) and Harley & Dransfield (2003), we measured pollen grain equatorial long axis (EL) and short axis (el) in polar view, for all symmetric ellipsoid grains, together with exine thickness, using a 100× oil immersion objective (producing a total 1000× magnification) and a micrometer eyepiece scale with an error of ± 1 μm (Figure 2A). The shape of the equatorial outline was calculated and expressed as the ratio EL/el, using a modification of the Erdtman (1969) shape class system introduced by Harley & Dransfield (2003). Images were captured using a Nikon Coolpix P3 digital camera. Pollen slide preparations have been deposited in the Palynological Collection at the Facultad de Ciencias, Universidad de la República (Montevideo, Uruguay).

2.2. *Scanning electron microscopy*

Fresh pollen grain material was mounted on aluminum stubs and sputter-coated with gold using an EMITECH K 550 sputter coater, and observed in a Jeol 5900 LV scanning electron microscope in N-SAT, Facultad de Ciencias, Universidad de la República (Montevideo, Uruguay).

2.3. *Pollen grain description*

Pollen descriptions were made based on the qualitative and quantitative characteristics observed from LM and SEM. Pollen grain size is described as 'small' (less than 30 μm in EL), 'medium' (30–50 μm in EL) or

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'large' (more than 50 μm in EL). Pollen grain terminology follows Punt et al. (2007).

Because of the sampling scheme in which individuals from each species were collected, and then 50 pollen grains from each individual were used, three sources of variation exist: among species, among individuals (within species), and among pollen grains (within individuals). To test the hypothesis of species being different, the variation among individuals (within species) should be used as the experimental error. To do this, we used a hierarchical linear mixed model including experimental error due to among-individual differences, and sub-sampling error due to variation among pollen grains within individuals. Differences among species were therefore tested with the experimental error degrees of freedom (i.e. the number of individuals within species) and not the sub-sampling error degrees of freedom. Thus, differences among species for each morphological variable (EL, el and their ratio) were compared with the following linear mixed model:

$$y_{ijj} = \mu + \alpha_i + \varepsilon_{ij} + \delta_{ijk} \quad (1)$$

where y_{ijk} is the response variable (i.e., EL, el or EL/el) of the i-th species, j-th individual sample, and k-th pollen grain; μ is the overall mean; α_i is the effect of the i-th species; ε_{ij} is the experimental error associated to the i-th species and the j-th individual sample; and δ_{ijk} is the sub-sampling error associated to the i-th species, j-th individual sample and k-th pollen grain. We assumed that $\varepsilon_{ij} \sim N(0, \sigma_e^2)$ and $\delta_{ijk} \sim N(0, \sigma_s^2)$. SAS Statistical Software (SAS Institute 2004) was used with the PROC MIXED procedure including species as a fixed effect for this analysis. When the analysis of variance (ANOVA) detected significant differences ($\alpha = 0.05$) among species, a Tukey ($\alpha = 0.05$) multiple comparison test was performed.

2.4. Pollen viability

Immediately after spathe opening, three flowers were collected from rachillae located in three different rachis zones: basal, medium and apical. Each rachilla was also divided into basal, medium and apical zones, thus collecting 27 flowers from each individual. Pollen grain viability was estimated by FCR, according to Heslop-Harrison & Heslop-Harrison (1970). Briefly, flowers were collected in the morning, right after anthesis, and pollen grains were extracted from each flower by shaking the anthers from one flower on a single slide. Then we added 20 μL of fluorescein diacetate dissolved in acetone (2 mg mL^{-1} stock solution) to a working solution concentration of 10^{-6} mol L^{-1} in 10% sucrose. The samples were incubated in a wet chamber for 10 minutes to allow for rehydration. They were then covered with a cover slip and analysed under the microscope. Viable (fluorescent) and non-viable (non-fluorescent) pollen grains were scored from images digitalised using an Olympus New Vanox AH-3 epiflourescence microscope and a Nikon DP71 digital camera. A total of 10 images were scored for each flower, with an average of 470 pollen grains scored per flower (range from 133 to 1136). Due to the need to discard one sample in some of the individuals because of loss of fluorescence, a final number of two of the three flowers from each rachilla and rachis zone were scored for each individual. Viability percentages were calculated for each species.

Since viability followed a binomial distribution, a generalised linear mixed model was used to compare viability among species. Statistical analyses were performed using SAS Statistical Software (SAS Institute 2004) with the PROC GENMOD (Generalized Linear Model) procedure. The model included species, rachis and rachilla zones, time between sample collection and image capture, and their interactions. A binomial distribution with the logit link function to obtain maximum likelihood estimates was used. To account for sub-sampling, an experimental error and a sub-sampling error were defined using the REPEATED statement (specifies the correlation structure and controls the displayed output from that model) for individuals within species.

3. Results

3.1. Palynological description

Based on the LM and SEM analyses, palynological descriptions of the four species of *Butia* studied are presented below (Table 2; Figure 2; Plates 1 and 2).

Butia odorata (Barb. Rodr.) Noblick. Pollen grains medium to large in size (EL from 50 to 79 μm), heteropolar, monosulcate. Shape ranges from symmetric to asymmetric perelliptic or pyriform in polar view. Exine tectate perforate, thickness 2.5 μm. Trichotomosulcate grains also occur, with a symmetric or asymmetric rounded triangular polar outline. The size range for the widest diameter of the trichotomosulcate grains is almost equivalent to that for the EL of the most frequent monosulcate grains. Both the elliptic and the triarmed sulci have rugulate aperture membrane (following Punt et al. 2007), which is destroyed by acetolysis. Plate 1, figures 1–3; Plate 2, figures 1–2.

Butia yatay (Mart.) Becc. Pollen grains large in size (EL from 52 to 79 μm), heteropolar, monosulcate. Shape ranges from symmetric to asymmetric elliptic or pyriform in polar view. Exine tectate perforate, thickness 2 μm. Aperture membrane rugulate is destroyed by acetolysis. Plate 1, figures 4–5; Plate 2, figures 3–4.

Table 2. Pollen morphological data for *Butia* species.

Taxon	AT	Size EL (μm)	Size el (μm)	Shape of equatorial outline	EL/el
B. odorata	Mono, trich	(50) 61 (79) a	(20) 29 (41) b	Perelliptic	2.11 ab
B. yatay	Mono	(52) 64 (79) a	(28) 36 (44) a	Elliptic	1.79 b
B. paraguayensis	Mono, trich	(31) 53 (78) ab	(16) 26 (43) bc	Perelliptic	2.17 a
B. lallemantii	Mono	(32) 45 (58) b	(13) 24 (34) c	Elliptic	1.95 ab

AT: aperture type, mono = monosulcate, trich = trichotomosulcate; size EL: size, long axis in μm, means (and ranges) in polar view; size el: size, short axis in μm, means (and ranges) in polar view; shape of equatorial outline and equatorial outline expressed as means of ratio EL/el. Same letters indicate no statistical differences between species ($\alpha = 0.05$). Data obtained from measurements and observations on 50 pollen grains per slide (individual) and two (*B. yatay*), three (*B. lallemantii*), four (*B. odorata*) or six (*B. paraguayensis*) individuals per species.

Butia paraguayensis (Barb. Rodr.) L.H. Bailey. Pollen grains medium to large in size (EL from 31 to 78 μm), heteropolar, monosulcate. Shape ranges from symmetric to asymmetric perelliptic or pyriform in polar view. Exine tectate perforate, thickness 2 μm. Trichotomosculcate grains also occur, with a symmetric or asymmetric rounded triangular polar outline. The size range for the widest diameter of the trichotomosulcate grains is almost equivalent to that for the EL of the most frequent monosulcate grains. Both the elliptic and the tri-armed sulci have a rugulate aperture membrane, which is destroyed by acetolysis. Plate 1, figures 6–8; Plate 2, figures 5–8.

Butia lallemantii Deble & Marchiori. Pollen medium to large in size (EL from 32 to 58 μm), heteropolar, monosulcate. Shape ranges from symmetric to

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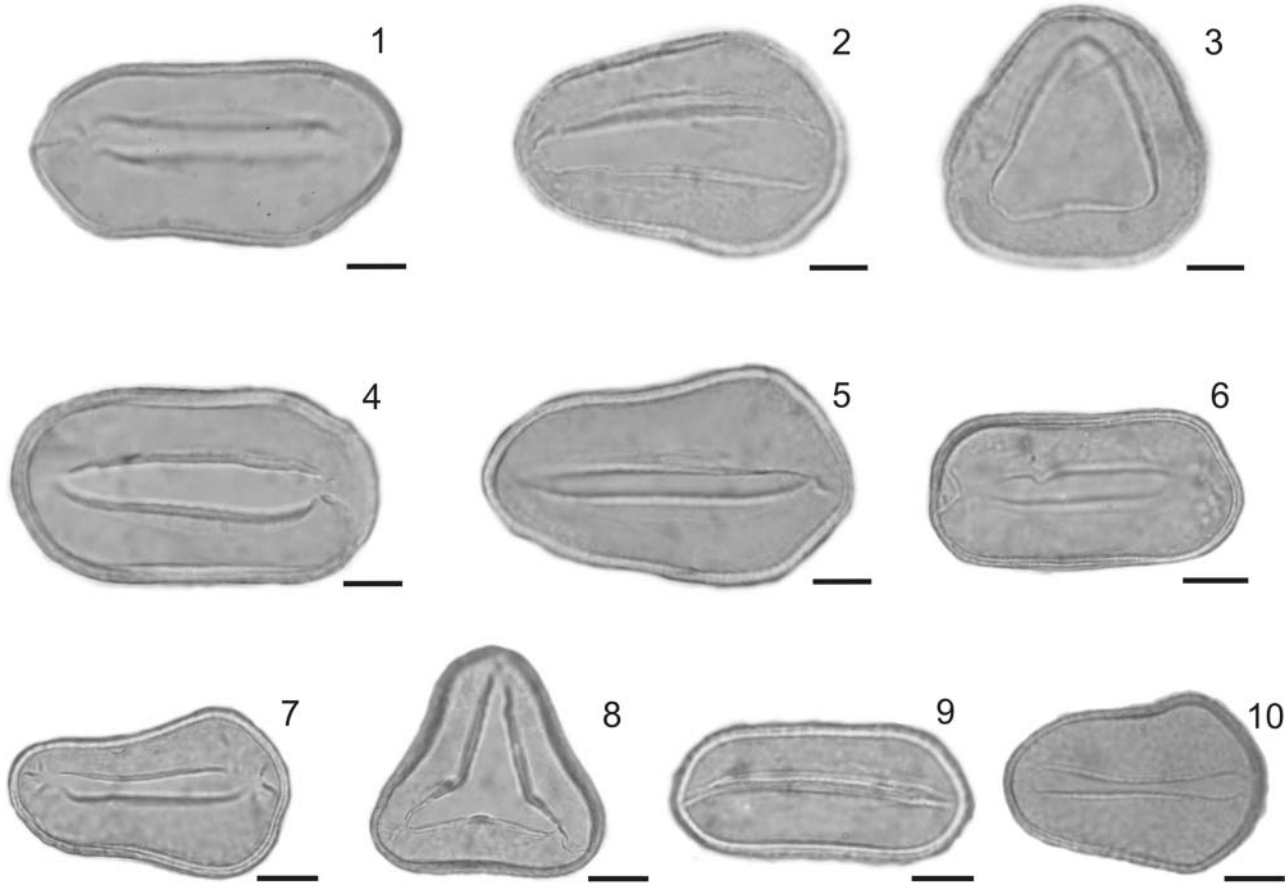


Plate 1. Light microscope micrographs of species of *Butia*. Acetolysed grains, polar view. 1. *B. odorata*, symmetric monosulcate grain. 2. *B. odorata*, asymmetric monosulcate grain. 3. *B. odorata*, trichotomosulcate grain. 4. *B. yatay*, symmetric monosulcate grain. 5. *B. yatay*, asymmetric monosulcate grain. 6. *B. lallemantii*, symmetric monosulcate grain. 7. *B. lallemantii*, asymmetric monosulcate grain. 8. *B. paraguayensis*, trichotomosulcate grain. 9. *B. paraguayensis*, symmetric monosulcate grain. 10. *B. paraguayensis*, asymmetric monosulcate grain. Scale bars: 10 μm.

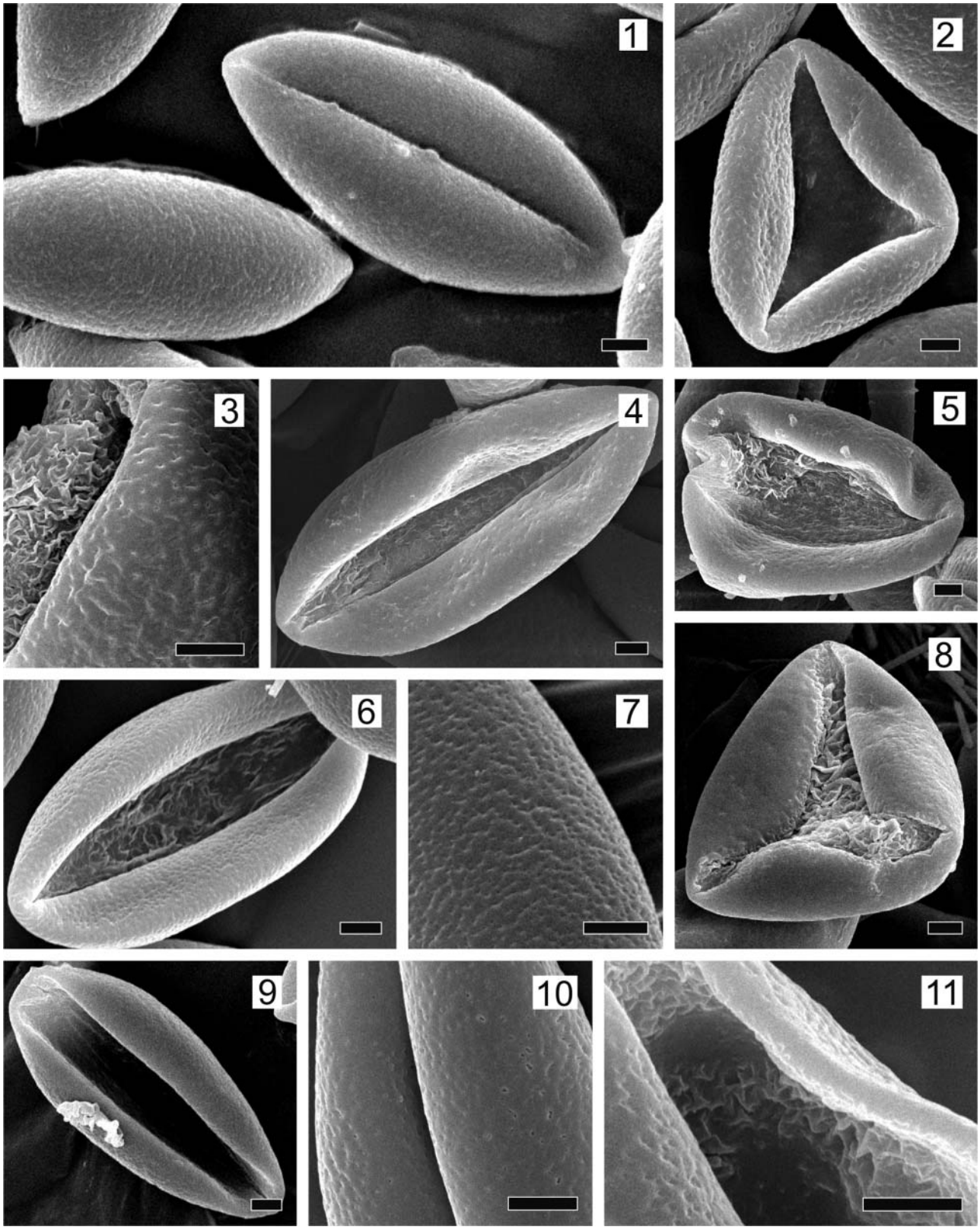


Plate 2. Scanning electron micrographs of species of *Butia*. Unacetolysed grains. 1. *B. odorata*, symmetric monosulcate grain, polar view, distal face. 2. *B. odorata*, trichotomosulcate grain, polar view, distal face. 3. *B. yatay*, detail of the perforated surface and of the rugulate aperture membrane. 4. *B. yatay*, asymmetric monosulcate grain, polar view, distal face. 5. *B. paraguayensis*, asymmetric monosulcate grain, polar view, distal face. 6. *B. paraguayensis*, symmetric monosulcate grain, rugulate aperture membrane, polar view, distal face. 7. *B. paraguayensis*, detail of the perforated surface, polar view, proximal face. 8. *B. paraguayensis*, trichotomosulcate grain, polar view, distal face. 9. *B. lallemantii*, symmetric monosulcate grain, polar view, distal face. 10. *B. lallemantii*, detail of the perforated surface, polar view, distal face. 11. *B. lallemantii*, detail of the perforated surface and of the rugulate aperture membrane. Scale bars: 5 μm.

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asymmetric elliptic or pyriform in polar view. Exine tectate perforate, thickness 2 μm. Rugulate aperture membrane is destroyed by acetolysis. Plate 1, figures 9–10; Plate 2, figures 9–11.

3.2. Pollen size differences

There are significant differences in pollen grains among the studied species in terms of lengths of EL and el, as well as for their ratio (Table 2; Figure 2). *B. yatay* and *B. odorata* have the largest pollen grains and *B. lallemantii* the smallest grains (Table 2). *B. paraguayensis* is intermediate in terms of pollen grain size (i.e. the length of its EL is not significantly different from the other three *Butia* species and the length of its el is not significantly different from that of *B. lallemantii and B. odorata*). However, its shape of the equatorial outline (ratio EL/el) is significantly different from that of *B. yatay*.

3.3. Pollen viability

Pollen grain viability of all *Butia* species ranged between 47 and 58% (Table 3; Plate 3). The highest pollen viability was obtained for the medium rachis zone, reaching up to 53%. *B. odorata* showed a higher average viability (58%). The four-way interaction between species, time between collection and capture, and rachilla and rachis zone was significantly different from zero ($p < 0.05$) for pollen viability. The most important effect was provided by the slope of decay in viability given by the time between collection and image capture (Table 4). *B. lallemantii* was the only species with a significant decay in pollen viability (Table 3), with both the apical and medium parts of the rachilla experiencing a significant decay while the basal part of the rachilla did not experience such decay.

4. Discussion

4.1. Differences in pollen grains morphology and size

All the species analysed produce monosulcate pollen grains, symmetric to asymmetric elliptic or pyriform in polar view, with a tectate perforate exine. However, *B. odorata* and *B. paraguayensis* produce both mono- and trichotomosulcate pollen grains. Harley & Baker (2001) consider that aperture number has an interesting systematic distribution within the family, with subfamily Arecoideae (to which *Butia* belongs) showing usually monosulcate pollen grains, occasionally combined with trichotomosulcate pollen grains, as observed in our study. Differences in aperture number among subfamilies have been associated with microsporogenesis type (Harley 1990). Areceaceae is one of the few families within the monocots in which both successive and simultaneous cytokinesis occur during microsporogenesis. The co-existence of both mono- and trichotomosulcate aperture types is the result of simultaneous cytokinesis, which normally results in tetrahedral tetrads in which cell plates progress centripetally (Harley & Baker 2001). Simultaneous cytokinesis in microsporogenesis seems to be an important element in the evolution of the angiosperms (Harley & Baker 2001), and therefore the trichotomosulcate condition would be indicative of a degree of specialisation in palms (Harley 1990). This could suggest incipient differentiation of *B. odorata* and *B. paraguayensis* with respect to *B. lallemantii* and *B. yatay*.

This diagnostic pollen grain characteristic could represent a feature of discrimination among populations within *Butia*. Some studies reported the co-ocurrence of both aperture types on some *Butia* species (Barth & Barbosa, 1971), while others reported only one type (i.e., monosulcate; Bauerman et al. 2010). We

Table 3. Viability data of pollen grains collected at the apical (AR), medium (MR) and basal (BR) parts of the rachis and from the apical (Ar), medium (Mr) and basal (Br) part of the rachillae.

	Viable pollen grains (%)													
	Rachis zone													
	AR				MR				BR					
Taxon	Ar	Mr	Br	**Av**	Ar	Mr	Br	**Av**	Ar	Mr	Br	**Av**	Average	Sample size
B. odorata	65	55	45	**56**	65	52	55	**59**	63	47	53	**56**	**58**	**20686**[1]
B. yatay	41	57	53	**50**	46	49	46	**47**	50	44	43	**46**	**47**	**31274**[2]
B. lallemantii	45*	51*	46	**47**	42*	53*	50	**50**	52*	51*	57	**53**	**51**	**29053**[1]
B. paraguayensis	57	58	48	**54**	52*	51	63	**55**	54	52	57	**54**	**54**	**28931**[1]
Average				**52**				**53**				**52**	**53**	

Av = average. Sample size = number of pollen grains scored per species. Zones where pollen grain viability is significantly influenced by the time spanned after sample collection are indicated with an asterisk.
[1]Data obtained from samples taken from three individuals per species.
[2]Data obtained from samples taken from four individuals per species.

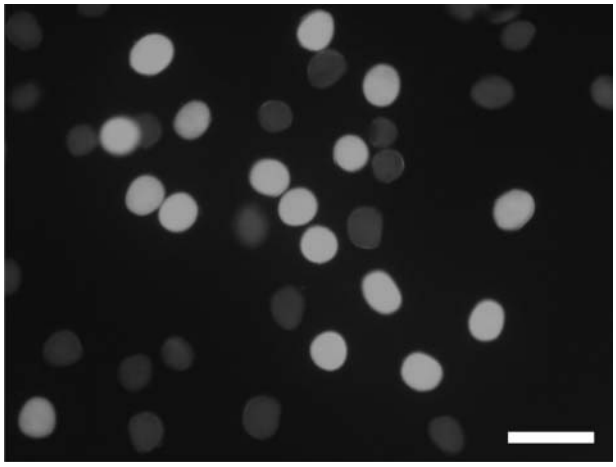


Plate 3. Pollen viability as fluorescent pollen grains in a germination medium containing fluorescein diacetate in acetone (2 mg mL^{-1}). Scale bar: 100 μm.

found both types of aperture, which could indicate a distinctive feature among Brazilian and Uruguayan populations. A record of *B. paraguayensis* on fossil pollen sequences from northeastern Uruguay or southern Brazil would be very interesting to resolve the question of whether there used to be a physical connection between the current population, disjunct areas or not. However, not finding its pollen grains in such a fossil pollen sequence does not necessarily imply it is absent from the vegetation, since a surface soil sample from the only site where a *B. paraguayensis* population occurs did not contain *Butia* pollen grains (Mourelle & Prieto 2012). This fact could be related to a low preservation potential of its grains.

Our results show significant differences in pollen grain size among the species of *Butia* included in this study. Statistical analyses suggest that the length of the short axis (el) and the shape of the equatorial outline are informative morphological characters to discriminate some species within *Butia*. *B. yatay* can be distinguished from all species in terms of size of el, whereas *B. odorata* can be separated from all except *B. paraguayensis* in terms of aperture. *B. paraguayensis* appears intermediate to *B. yatay* and *B. lallemantii* in all dimensions measured, and can be distinguished by the presence of trichotomosulcate pollen grains and the shape of the equatorial outline. This intermediate condition was already found for genetic variation in natural populations assessed through neutral molecular markers, in which *B. paraguayensis* showed high genetic proximity to the two other species, preventing the three taxa from being considered clearly separate genetic entities (Gaiero et al. 2011). We suggest that pollen grain size should be used together with pollen aperture type and other highly informative morphological markers such as the ones used by Marcato (2004) – habit, stem height, fruit shape, inflorescence length and number of rachillae per inflorescence – to assist in proper species delimitation and individual assignment to a specific entity, an essential step in the first stages of conservation management (Frankham et al. 2002).

Pollen grain size should be used with care when comparisons are made across studies, because there are several factors that could affect this variable. Firstly, pollen grain samples are usually processed according to standard palynological techniques which may affect pollen grain size variation (Reitsma 1969; Dickson 1988; Harley & Baker 2001; Schüler & Behling 2011). Secondly, the mounting medium can affect pollen grain size. Glycerol jelly tends to produce larger grains than silicone oil due to grain swelling (Moore & Webb 1978). In our case, we reduced the time in which the grains were immersed in glycerol by measuring them on the same day they were mounted, thus reducing their swelling. Thirdly, we have found that the type of material studied also seems to affect pollen grain size. Bauermann et al. (2010) obtained pollen grain samples from herbarium material and observed EL sizes for *B. yatay* approximately 10 μm smaller than in our study of fresh material, although this difference can also be due to interpopulation variability within the species.

Table 4. Slope of viability decay with the time spanned after sample collection calculated through a generalised linear mixed model for the apical (AR), medium (MR) and basal (BR) parts of the rachis and the apical (Ar), medium (Mr) and basal (Br) part of the rachillae. Decays significantly larger than zero are indicated with an asterisk.

	Rachis zone								
	AR			MR			BR		
Taxon	Ar	Mr	Br	Ar	Mr	Br	Ar	Mr	Br
B. odorata	−0.273	−0.053	-0.029	−0.187	−0.212	−0.179	−0.111	0.003	0.063
B. yatay	−0.133	−0.083	0.000	0.107	0.000	0.023	0.148	0.107	0.133
B. lallemantii	−0.303*	−0.295*	-0.236	−0.217*	−0.226*	−0.221	−0.189*	−0.194*	−0.097
B. paraguayensis	−0.133	−0.199	-0.129	−0.057	−0.150	−0.107	−0.027	0.055	0.051

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4.2. Pollen viability

Pollen viability measured in *Butia* species was between 47 and 58%, a value considered acceptable compared to similar values found in economically important palm species as *Cocos nucifera* L. and *Phoenix dactylifera* L. (Ismail & Zohair 2013; Machado et al. 2014). Pollen viability of all species of *Butia* analysed did not seem to be a limiting factor to reproductive success in terms of potential fertilisation success and seed production (Aizen & Harder 2007). Therefore, regeneration problems due to recruiting should be addressed to ensure their demographic recovery. Habitat fragmentation can affect connectivity among populations (via reduction on pollen or seed dispersal), leading to genetic structure and population differentiation (tested by Damschen et al. 2014 for seed dispersal). Gaiero et al. (2011) have shown that this is not the case in the same *Butia* populations included in this study, but their data were collected from adult individuals, so they might be reflecting a situation prior to the fragmentation events.

The proportion of viable pollen grains was not significantly different along the rachis and rachillae, so pollen grains taken from any zone in the inflorescence can be used for in-vitro pollen germination and fertilisation studies. However, it is important to bear in mind that samples should be processed and analysed immediately after collection to produce a more accurate estimation of pollen viability, since our results showed that time elapsed after collection had a high impact on viability, producing a decay that may be underestimating the actual pollen viability of an individual, especially in certain species and zones. A rapid decline of pollen viability might affect effective pollination negatively, especially in the case of species that seem to depend on cross-pollination (Dafni & Firmage 2000). This becomes more relevant in small or fragmented populations, since this decline could mean reduced gene flow via pollen grains between distant populations. However, rapid decline might be a strategy to prevent self-pollination, by making optimum pollen germinability out of phase with the time of stigma receptivity (Dafni & Firmage 2000).

5. Conclusions

We have found morphological characters which are informative for species delimitation in *Butia* (pollen grain aperture, pollen grain equatorial short axis length and shape of the equatorial outline), and show that the nomenclature used so far is suitable for the species included in this study. Our results on pollen viability have implications for species conservation and for future research. Pollen viability was high (up to 58%), so pollen quality is not a limiting factor to the regeneration of Uruguayan populations of *Butia*, but measures should be taken to ensure effective pollination takes place in spite of fragmentation in their natural habitats. This variable is highly affected by the time elapsed between the collection of samples and their processing, and so samples should be evaluated shortly after collection to get accurate estimations.

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