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Complex sporoderm structure in bryophyte spores: a palynological study of Erpodiaceae Broth.

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ABSTRACT

Palynological studies of bryophytes are critical for evaluating the taxonomic relevance of their spores. They also provide important support to paleoecological investigations that, usually, treat bryophytes as a whole, which does not permit the evaluation of specific functional traits of a special taxonomic unit. The present study investigated the morphology and ultrastructure of spores of five species of Erpodiaceae (Bryophyta), and assessed the implications for taxonomy and the recognition of spores of past records. Erpodiaceae includes corticolous and saxicolous plants that are widely distributed throughout tropical and temperate regions. The spores were found to be isomorphic and apolar with a subcircular amb, granulate, inaperturate. The sporoderm possesses a perine, an exine and a stratified intine. The perine is largely responsible for spore surface ornamentation. The occurrence of exine projections, in isolation or sustaining the elements of the perine, characterizes sporoderm structure with features similar to that of a semitectum, a distinctive characteristic that has not been reported previously for bryophyte spores.

Keywords: bryophytes, mosses, palynology, spore, sporoderm, ultrastructure

Introduction

The moss family Erpodiaceae Broth. is widely distributed in tropical and temperate regions. The plants are small, prostrate, grow as mats and are autoicous and cladocarpous. Their stems are creeping and irregularly pinnate, while the leaves are oblong to oblong-ovate. They lack costa, have smooth or pluripapillose laminal cells, usually short seta and a capsule that is immersed to exserted (Pursell 1994; Pursell & Allen 2002; Faria *et al.* 2018).

According to the classifications proposed by Frey & Stech (2009) and Goffinet *et al.* (2009), Erpodiaceae includes five genera: *Aulacopilum*, *Erpodium*, *Solmsiella*, *Venturiella*, and *Wildia*.

Costa *et al.* (2011) and Yano (2011) report seven species of Erpodiaceae for Brazil, whereas Faria *et al.* (2018) consider there to be five.

The species occur on tree bark and branches (Vital 1980; Pursell & Allen 2002) in forests and savannas, including the Cerrado and Caatinga, but particularly in areas having anthropic influences (Vital 1980; Faria *et al.* 2018).

Vital (1980) points out that species of Erpodiaceae occur in areas where vegetation is sparse, even when in forests, where they receive high luminosity. Their forophytes typically have a corky bark.

Little is known about the spores of species of Erpodiaceae. Erdtman (1965) described spores of *Erpodium beccarii* (as *Erpodium lorentzianum*), while other sparse information about spore size or surface ornamentation has been reported in various taxonomic studies (Pursell 1966; 1994; Crum

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1972; Yano 1984; Yano & Santos 1993; Stone 1997; Pursell & Allen 2002; Daniels *et al.* 2012).

The sporoderm of mosses is known to include three strata: intine, exine and perine (Olesen & Mogensen 1978; Neidhart 1979; Mogensen 1981; 1983). The perine may represent the only ornamented sporoderm stratum which has been considered a synapomorphy of mosses and tracheophytes (Mishler & Churchill 1984; Bremer *et al.* 1987).

Spore morphology and ontogeny are important for evaluating bryophytes taxonomy and phylogeny, as evidenced by several studies (Clarke 1979; Horton 1982; Mishler & Churchill 1984; Blackmore & Barnes 1987; Brown & Lemmon 1980; 1984a; b; 1988; 1991; Estébanez *et al.* 1997; Luizi-Ponzo *et al.* 1997; Caldeira *et al.* 2006; 2009; 2013; Luizi-Ponzo & Melhem 2006a; b; Yano & Luizi-Ponzo 2006; 2011; Savaroglu *et al.* 2007; Rocha *et al.* 2008; Savaroglu & Erkara 2008; Alfayate *et al.* 2013; Brown *et al.* 2015; Rodrigues & Luizi-Ponzo 2015; Savaroglu 2015; Savaroglu *et al.* 2017; Silva-e-Costa *et al.* 2017). Nonetheless, data are still scarce for Erpodiaceae (Erdtman 1965), or have been reported in taxonomic studies (Pursell 1966; 1994; Crum 1972; Yano 1984; Yano & Santos 1993; Stone 1997; Pursell & Allen 2002; Daniels *et al.* 2012).

The present study aimed to provide a detailed account of the morphology and ultrastructure of the spores of five species of Erpodiaceae from Brazil, in order to evaluate the taxonomic importance of spore morphology for the family and to facilitate their identification in past palynological records.

Materials and methods

Studied specimens and palynological treatments

The following species were selected for palynological study after an extensive review of herbaria material: *Aulacopilum glaucum* Wilson, *Erpodium beccarii* Müll. Hal., *Erpodium coronatum* (Hook. f. & Wilson) Mitt., *Erpodium glaziovii* Hampe, and *Erpodium pringlei* E. Britton. The specimens used in the present study were obtained from the following herbaria: Herbarium Maria Eneyda P. K. Fidalgo of the Institute of Botany of São Paulo (SP), Herbarium of the Departament of Botany of the Museu Paraense Emílio Goeldi (MG) and Herbarium of the Botanical Garden of Rio de Janeiro (RB) (acronyms according to Thiers 2012).

Examined material

Aulacopilum glaucum Wils.: Brazil. Mato Grosso: Antônio João, 25/V/1976, D. Vital 6457 (SP*); Paraná: Clevelândia, 15/I/1983, O Yano et al. 5547 (RB); Paraná: Rio Negro, 28/I/1983, O. Yano & J. R. Pirani 7134 (SP); Rio Grande do Sul: Condor, 19/I/1983, O. Yano et al. 5688 (SP); Rio Grande do Sul: Panambi, 20/I/1983, O. Yano & J. R. Pirani 5692 (SP); Santa Catarina: Chapecó, 21/IV/1983, O. Yano & J. R. Pirani 6640 (SP); Santa Catarina: Xanxerê, 21/IV/1983, O. Yano & J. R. Pirani 6600 (SP); São Paulo: Bauru, 8/XII/1978, D. M. Vital 8391 (SP); São Paulo: Mirante do Paranapanema, 9/III/1981, O. Yano 3246 (SP). *Erpodium beccarii* Müll. Hal.: Brazil. Paraná: Maringá 2/X/1989, O. Yano 13433 (SP); Paraná: Medianeira 22/III/1982, O. Yano 4104 (SP*); São Paulo: Mirassol 17/VII/1892, O. Yano & T. Yano 4442 (SP); São Paulo: Prataria, 8/VI/1985, O. Yano & T. Yano 9494 (SP). *Erpodium coronatum* (Hook. & Wils.) Mitt. Brazil. Mato Grosso: Guia 18/VI/1981, D. M. Vital 9950 (SP); Mato Grosso do Sul: Bonito 28/XI/1979, D. M. Vital 8588 (SP); Pernambuco: Inajá, 5/IX/1980, O. Yano & Andrade Lima 2915 (SP); São Paulo: Dracena 25/VIII/1983, O. Yano & R. C. Compagnoli 8289 (SP); São Paulo: Fernandópolis 20/VII/1982, O. Yano & T. Yano 4495 (SP*); São Paulo: Fernandópolis O. Yano & T. Yano 4500 (SP); São Paulo: General Salgado 21/VII/1982, O. Yano & T. Yano 4507 (SP). *Erpodium glaziovii* Hampe: Brazil. Espírito Santo: Aracruz 22/XI/1982, O. Yano et al. 4864 (SP); Espírito Santo: Colatina 23/XI/1982, O. Yano et al. 4890 (SP); Espírito Santo: Ibiraçu 22/XI/1982, O. Yano et al. 4857 (SP*); Minas Gerais: Divino 24/XI/1982, O. Yano et al. 4975 (SP); Rio de Janeiro: Macaé 20/XI/1982, O. Yano et al. 4729 (SP); São Paulo: Guaratinguetá 18/XI/1982, O. Yano et al. 4711 (SP); O. Yano et al. 4713 (SP); São Paulo: Itu, 9/IX/1990, A. Schäfer-Verwimp 13152 (MG). *Erpodium pringlei* E. Britton: Brazil. Minas Gerais: Campanha 20/III/1983, O. Yano & D. P. Santos 6300 (RB); Paraná: Jacarezinho 23/VIII/1983, O. Yano & R. C. Compagnoli 8195 (SP*); Paraná: Maringá 16/III/1982, O. Yano 3961 (SP); Paraná: Terra Roxa 22/III/1982, O. Yano 4088 (SP); São Paulo: Águas de Lindóia, 8/IV/1990, O. Yano & Z. R. de Melo 14459 (SP); São Paulo: Mirante do Paranapanema, 9/III/1981, O. Yano 3248 (SP); São Paulo: São João da Boa Vista 22/III/1982, D. M. Vital 10357 (SP).

Qualitative and quantitative analysis of spores were performed under light microscopy (LM) before and after acetolysis following Wodehouse (1935) and Erdtman (1960), respectively. Both techniques were adapted to bryophytes according to Luizi-Ponzo & Melhem (2006a). Palynological terminology follows Punt *et al.* (2007).

For scanning electron microscopy (SEM), spores were attached to specimen stubs that were previously covered with double-sided tape, and then sputter coated with a 20 nm layer of gold. For transmission electron microscopy (TEM), spores were fixed in 2.5 % glutaraldehyde and rinsed four times in a buffer solution. Post-fixation was performed in 1 % osmium tetroxide, followed three-hours later by dehydration in an alcohol series, embedding in Spurr resin, and heating at 70 °C for 48 hours. Ultrathin sections (65-70 nm) were cut and then stained with uranyl acetate and lead citrate (Reynolds 1963).

Statistical analysis

A minimum of four specimens was observed for each species. Mean values for spore diameter were calculated from measurements under LM of 100 spores for the reference specimen (RS; indicated by an * in Specimens Investigated List and the Tables), and 30 spores for comparison specimens (CS). Means (M), standard deviations (S), standard errors (S_M), ranges (X_{min}-X_{max}), confidence intervals (CI), and variability (V) are reported. Box-plot graphics were used to illustrate morphometric variation.

Measurements of each sporoderm stratum (perine, exine, and intine) from non-acetolyzed spores, and exine thickness from acetolyzed spores, were based on 10 spores per sample.

Measurements were submitted to the Shapiro-Wilk normality test and graphic evaluation, which revealed the data to be non-parametric (not normally distributed) (p value= 1.338^{-12}). Thus, the non-parametric Kruskall-Wallis test was used to test difference among and between data, followed by Dunnet's test (a posteriori analysis) to identify differences. Medians and data distribution were evaluated graphically. Statistical analysis and graphing were performed using R 3.5.1 (R Core Team 2018) and JMP®, version 12 (SAS Institute, Cary, North Carolina, USA).

Results

Spores of the studied species of Erpodiaceae were found to be isomorphic (Fig. 1A-L), small to medium in size (13.20 to 39.60 µm, Fig. 2A), apolar, subcircular, inaperturate (Fig. 1A-F), and granulate (Fig. 1A, B, D, H, I, Tab. 1). There was no evidence of internal or external polarity of the spores (Fig. 1C, E, F).

Analysis by LM (Fig. 1B, F, G) and SEM (Fig. 1H, I) revealed that the elements of the perine consist of irregularly shaped and sized granules that occurr in isolation, overlapping or united, to form small walls (Fig. 1 A, C, D, H, I). Analysis by TEM revealed the perine to be more electron-dense than the exine, with its elements often exhibiting a very elaborate structure, such as a semitectum (Fig. 1J-L).

The exine was observed to be thin (Tab. 2), not uniformly shaped, and broken in some regions of the spore, under different preparations, but especially after acetolysis (Fig. 1A). TEM revealed the exine to be less electron-dense than the perine. The exine exhibits sharp and discrete projections, which sustain the elements of the perine (Fig. 1K-L).

The intine was observed to be thick (Tab. 2) and stratified, with a shiny interior region and a denser more-opaque external region (Fig. 1F, G, K, L). No particular pattern was observed in the thickness of the sporoderm, and it did not accompany any morphological changes in the other strata (Fig. 1B, E, F, G). TEM revealed the intine to have a fibrillar

Table 1. Morphometric data for spore diameter (µm) of the five studied species of Erpodiaceae. * reference specimen (RS) n=100; comparison specimens (CS) n=30.

Material	(X_{min}-X_{max}) M±s_M		s	CI (95 %)	V (%)
Aulacopilum					
A. glaucum Wilson D. M. Vital 6457 *(RS)	(19.80-28.60)	25.39 ±±0.23	2.33	24.93-25.85	9.18
O. Yano & J. R. Pirani 6600(CS)	(17.60-26.40)	23.03±±0.38	2.06	22.26-23.80	8.94
O. Yano & J. R. Pirani 7134(CS)	(19.80-26.40)	23.91±±0.38	2.06	23.14-24.68	8.62
O. Yano & J. R. Pirani 6640(CS)	(17.60-24.20)	21.27±±0.35	1.94	20.54-22.00	9.12
O. Yano *et al.* 5547(CS)	(15.40-19.80)	17.60±±0.32	1.73	16.95-18.25	9.83
Erpodium					
E. beccarii Müll. Hal. O. Yano 4104 *(RS)	(24.20-37.40)	30.62 ±±0.25	2.48	30.13-31.11	8.10
O. Yano & T. Yano 4442(CS)	(26.40-37.40)	32.78±±0.52	2.85	31.72-33.84	8.69
O. Yano & T. Yano 9494(CS)	(24.20-35.20)	29.85±±0.61	3.35	28.60-31.10	11.22
O. Yano 13433(CS)	(19.80-28.60)	23.39 ±±0.51	2.80	22.35-24.43	11.97
E. coronatum (Hook. f. & Wilson) Mitt. O. Yano & T. Yano 4495 *(RS)	(28.60-39.60)	33.84±±0.29	2.88	33.27-34.41	8.51
O. Yano & T. Yano 4507(CS)	(30.80-39.60)	34.91±±0.48	2.63	33.93-35.89	7.53
O. Yano & R. C. Compagnoli 8289(CS)	(26.40-37.40)	31.02±±0.61	3.34	29.77-32.27	10.77
D. M. Vital 9950(CS)	(28.60-39.60)	35.42±±0.51	2.79	34.38-36.46	7.88
O. Yano & T. Yano 4500(CS)	(28.60-37.40)	32.93±±0.49	2.68	31.93-33.93	8.14
E. glaziovii HampeO. Yano *et al.* 4857 *(RS)	(15.40-26.40)	19.51 ±±0.21	2.11	19.09-19.93	10.80
O. Yano *et al.* 4864(CS)	(19.80-28.60)	23.32±±0.44	2.42	22.42-24.22	10.38
O. Yano *et al.* 4711(CS)	(13.20-28.60)	23.32±±0.70	3.81	21.90-24.74	16.34
O. Yano *et al.* 4729(CS)	(17.60-30.80)	24.27±±0.66	3.63	22.91-25.63	14.96
A. Schäfer-Verwimp 13152(CS)	(22.00-30.80)	25.81±±0.47	2.58	24.85-26.77	10.00
E. pringlei E. Britton O. Yano & R. C. Compagnoli 8195 *(RS)	(19.80-28.60)	25.85 ±±0.24	2.43	25.37-26.33	9.40
O. Yano & D. P. Santos 6300(CS)	(19.80-28.60)	24.27 ±±0.43	2.35	23.39-25.15	9.68
O. Yano & Z. R. de Melo 14459(CS)	(22.00-30.80)	25.37±±0.42	2.29	24.51-26.23	9.03
D. M. Vital 10357(CS)	(17.60-28.60)	23.98 ±±0.49	2.67	22.98-24.98	11.13

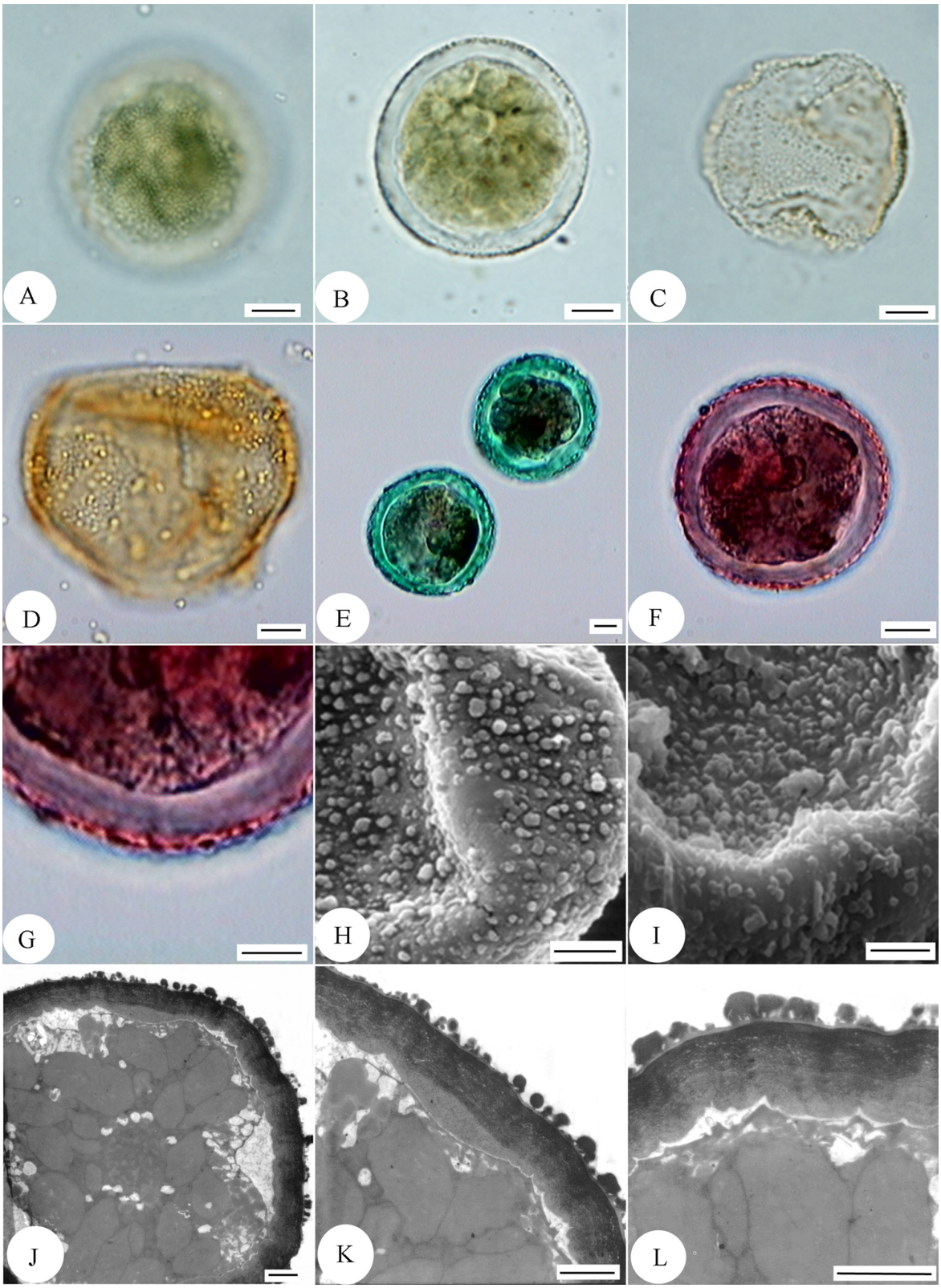

Figure 1. A-L. Spores of studied species of Erpodiaceae. **A-C.** Spores of *Aulacopilum glaucum*. **A.** Surface view of non-acetolyzed spore (LM). **B.** Sporoderm view of non-acetolyzed spore (LM). **C.** Surface view of acetolyzed spore (LM). **D.** Spores of *Erpodium coronatum*. Surface view of acetolyzed spore (LM). **E-I.** *Erpodium beccarii* **E.** General view of two non-acetolyzed spores (LM). **F.** Sporoderm view of non-acetolyzed spore (LM). **G.** Sporoderm detail of non-acetolyzed spore (LM). **H.** Sporoderm surface (SEM). **I.** Sporoderm detail (SEM). **J-L.** *Erpodium pringlei*. **J.** General view of non-acetolyzed spore (TEM). **K.** Detail of sporoderm of non-acetolyzed spore (TEM). **L.** Detail of sporoderm showing exine columns and stratified intine of non-acetolyzed spore (TEM). Scale bars: A-F: 5 µm G-L: 2 µm.

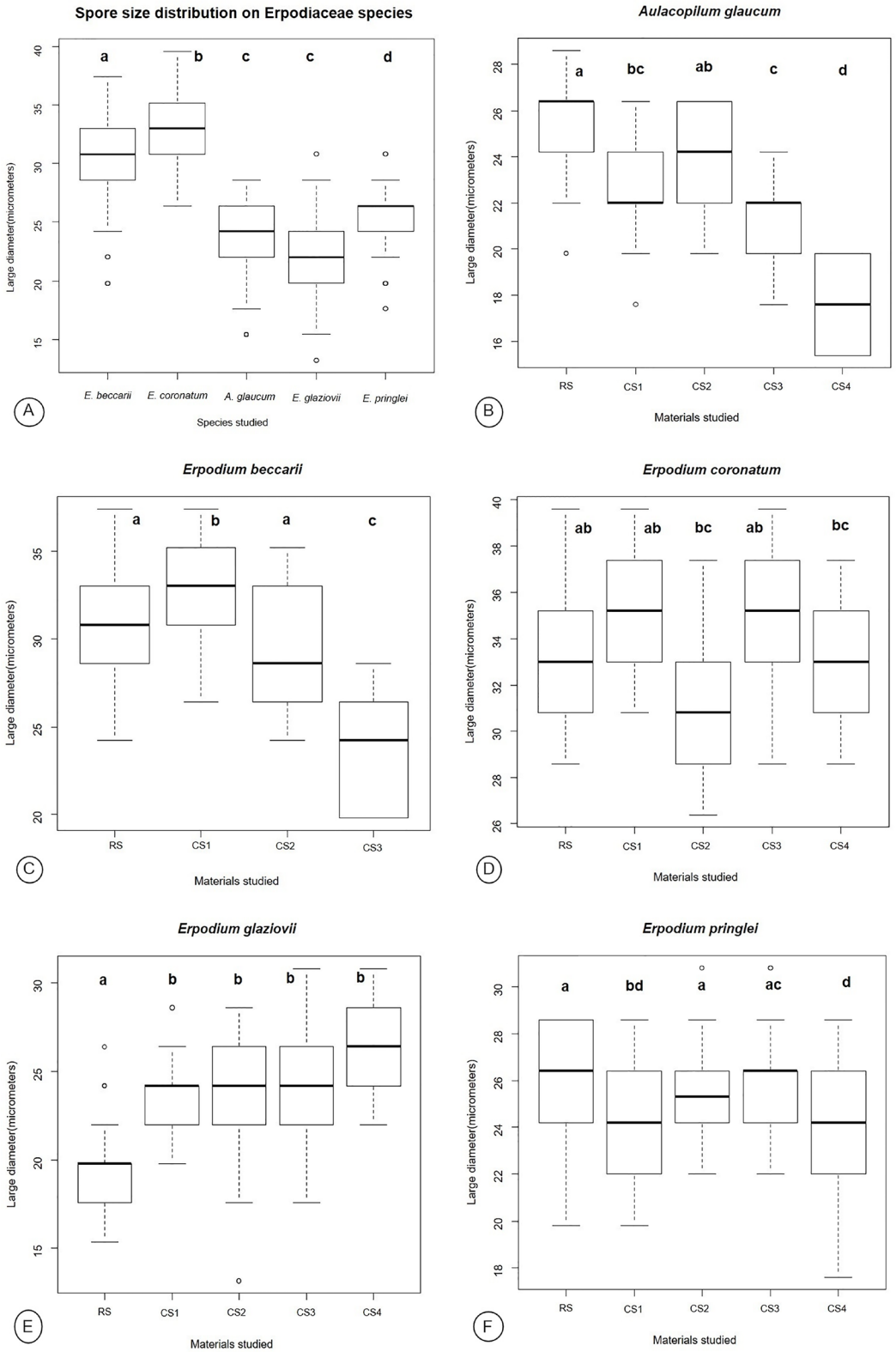

Figure 2. A-F. Boxplots representing spore size distribution for the five studied species of Erpodiaceae. **A.** Interspecific comparison of spore size. **B.** Intraspecific comparison of spore size for *A. glaucum*. **C.** Intraspecific comparison of spore size for *E. beccarii*. **D.** Intraspecific comparison of spore size for *E. coronatum*. **E.** Intraspecific comparison of spore size for *E. glaziovii*. **F.** Intraspecific comparison of spore size for *E. pringlei*. Error bars above and below the boxes indicate the 90th and 10th percentiles, while white circles represent outliers. Different letters (a, b, c, d) represent statistical differences among the specimens studied (i.e., specimens that do not have the same letters are statistically different; Kruskall-Wallis test and Dunnet's test, $p < 0.05$).

appearance, to be more compact in the area adjacent to the exine, and to possess stratification (Fig. 1J-L).

Observations demonstrated spore mortality to be low, and this indicates the absence of any phenomenon related to aborted spores. Kruskall-Wallis and Dunnet's tests revealed a statistically significant difference among species for the specimens analyzed ($p < 0.0001$ for both tests) (Fig. 2A). Statistically significant intra-specific variation was also detected for all species studied ($p < 0.0001$ for *A. glaucum*, *E. beccarii*, *E. coronatum*, and *E. glaziovii*; $p = 0.0003$ for *E. pringlei*:) (Fig. 2B-F). Inter- and intraspecific variation precluded distinguishing the studied species based on spore size.

Table 2. Mean thickness (μm) of sporoderm strata for spores of of the five studied species of Erpodiaceae, before and after# acetolysis.

Species	Sporoderm strata			
	exine#	exine	intine	perine
Aulacopilum glaucum Wilson D. M. Vital 6457 *(RS)	0.46	0.48	2.32	0.54
Erpodium beccarii Müll. Hal. O. Yano 4104 *(RS)	0.47	0.52	2.50	0.64
E. coronatum (Hook. f. & Wilson) Mitt. O. Yano & T. Yano 4495 *(RS)	0.45	0.46	2.84	0.66
E. glaziovii Hampe O. Yano *et al.* 4857 *(RS)	0.49	0.52	2.44	0.58
E. pringlei E. Britton O. Yano & R. C. Compagnoli 8195 *(RS)	0.42	0.44	2.60	0.70

Discussion

Previous descriptions of the spores of some species of Erpodiaceae (Pursell 1966; 1994; Yano & Santos 1993; Daniels *et al.* 2012) revealed differences in size and slight variation in surface ornamentation. The surface ornamentation was characterized as "papillose", except for *Erpodium coronatum* for which the spores were considered essentially smooth (Pursell 1966; 1994; Yano & Santos 1993). The present study found the spores of this species to have the most delicate ornamentation among those studied.

Crum (1972) referred to spores of Erpodiaceae as "relatively large (18-44 μm), and scabrate, nearly smooth". This variation in size corresponds to the small and medium size classes of Erdtman (1952), which is in agreement with the present study.

When describing spores of *Erpodium beccarii* (as *Erpodium lorentzianum*), Erdtman (1965) reported an inaperturate condition and a surface with circular or irregularly shaped processes. These features are similar to those found for the species of the present study. However, it is worth noting that, according to Erdtman (1965), the exine is the stratum responsible for spore ornamentation, while the present study found the perine to be responsible, which is consistent with the observations of McClymont & Larson (1964), Olesen & Mogensen (1978), Neidhart (1979) and Brown & Lemmon (1980; 1984a; 1988), and more recent papers on moss spores (Caldeira *et al.* 2013; Rodrigues & Luizi-Ponzo 2015; Savaroglu 2015; Savaroglu *et al.* 2017).

The perine totally or partially covers the exine and is thus largely responsible for spore surface ornamentation. The electron density of the perine, and its resistance to acetolysis, indicated it to be composed of lipidic and sporopollenin material, as also reported for moss species by Neidhart (1979).

Observations under TEM revealed the presence of projections from the exine that occur in isolation or sustain the elements of the perine. Sporoderm structure with features similar to the semitectum of pollen grains has not been previously reported for spores of mosses in the literature consulted and thus represents an important morphological character evidencing the close relationship of these plants to tracheophytes, as previously pointed out by some authors, but especially Mishler & Churchill (1984), who included perine ontogeny in their phylogenetic analysis.

Intine thicknesses was found to be not associated with exine thickness in the species of the present study. This observation, associated with the irregular rupture of the exine while submitted to acetolysis, confirms the absence of an apertural area and the inaperturate, and apolar condition of these spores. Although an aperture at the proximal pole is common in mosses, the absence of an apertural region has been reported for spores of the haplolepidous moss *Fissidens crispus* (as *Fissidens limbatus*) by Mueller (1974) and for some spores of Grimmiaceae by Estébanez *et al.* (1997). It is interesting to note that the apolar condition reported here for spores of Erpodiaceae demonstrates the absence of internal polarity, as also observed by Mueller (1974) for species of *Fissidens*. On the other hand, Estébanez *et al.* (1997) found evidence of spore polarity for species of Grimmiaceae.

The intine stratification found for the studied species indicates a relationship with the arrangement of the fibrils, which are more compact in the area adjacent to the exine. Such stratified condition of the intine has also been reported for haplolepidous mosses, such as Fissidentaceae (Mueller 1974), and Grimmiaceae (Estébanez *et al.* 1997), as well as for taxa considered more distant, such as the cleistocarpous family Archidiaceae, in which the stratified intine possesses a great number of layers (McClymont & Larson 1964; Miles & Longton 1992). Luizi-Ponzo & Melhem (2006a) reported stratified intine for spores of Helicophyllaceae, evidencing heterogeneity in the distribution of this feature among mosses.

The palynological observations reported here do not permit separating neither the genera nor the species of Erpodiaceae, but can be considered to reflect a common evolutionary pattern for the family, especially with regard to the configuration of the perine and intine. These results

represent information that can be of important relevance to future phylogenetic analyses, as there was no consensus among the authors on previous studies (De Luna 1995; Goffinet *et al.* 1998). The spores of the studied species were resistant to acetolysis, and so their occurrence in historical records can be expected.

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