Spores of *Plagiochila* (Dumort.) Dumort.: the taxonomic relevance of morphology and ultrastructure

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ABSTRACT

Plagiochilaceae is a family of leafy liverworts that are distributed worldwide. It is of great importance due to its taxonomic and ecological implications among bryophytes. Most species of the family belong to the genus *Plagiochila*, but there is no consensus regarding its infrageneric circumscription. There have been few palynological studies involving Plagiochilaceae and *Plagiochila*. Here, we describe the spore morphology of seventeen species of *Plagiochila* and discuss the taxonomic value of palynological characters for these taxa. The spores were processed by standard palynological techniques and analyzed using light and electron microscopy. The spores were found to be apolar, spheroidal, released monads that vary in size from 13μm to 58μm (small to large size). The sporoderm comprises an intine (stratified), a nexine, and a sexine. The spore surface is ornamented with granules that vary in shape and morphology, thus allowing the studied species to be grouped into four spore types: regular and delicate granulate, irregular and coarse granulate, long granules with flattened apices, and long and straight granules. Hierarchical cluster analysis revealed five different groups of species, evidencing the importance of spore information for taxonomic and phylogenetic studies.

Keywords: bryophytes, liverworts, morphology, palynology, SEM, taxonomy, TEM, ultrastructure

Introduction

Plagiochilaceae (Lophocoleineae) is a family of leafy liverworts that includes robust, ascending or pendent plants (Heinrichs 2002; Gradstein & Costa 2003). Their leaves are alternate or opposite, succubous and with a ciliated or entire reflexed dorsal margin, while underleaves are generally absent (Gradstein *et al.* 2001; Gradstein & Costa 2003). Plagiochilaceae is an important group of bryophytes in the tropics due to its high species richness (Gradstein & Reiner-Drehwald 1995), but it is also diverse in subtropical and temperate regions (Jamy *et al.* 2016). The family contains ten genera that are distributed worldwide (Crandall-Stotler *et al.* 2009; Söderström *et al.* 2013; 2016), with *Plagiochila* being the richest by far with ca 96 % of the species of the family (Gradstein *et al.* 2001; Heinrichs 2002; Gradstein & Costa 2003; Jamy *et al.* 2016; Söderström *et al.* 2016).

Plagiochila is a taxonomically complex group, with more than 2300 published names (Inoue 1989), and maybe as many as approximately 3000 names (ELPT database). So & Grolle (2000) reported ca 450 species distributed worldwide, whereas Gradstein (2015a; b; 2016) reviewed and contributed to synonymization and lectotypification of several names, and so more recent estimates place the number of currently accepted species at 700 (Söderström *et al.* 2016).

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Numerous attempts at infrageneric classification of *Plagiochila* have been made, with early attempts relying on some important characters of the gametophyte, such as leaf shape, leaf cell pattern and branching type (Lindenberg 1839; Spruce 1885; Schiffner 1900; Stephani 1902; Dugas 1929; Carl 1931). Later, studies of Schuster (1959; 1960), Inoue & Schuster (1971), and more recently by So (2001), and Hässel (2004; 2006), used gametophyte morphology for the classification of species of *Plagiochila*. Molecular phylogenetic studies of *Plagiochila* and Plagiochilaceae (Heinrichs 2002; Groth *et al*. 2003; Patzak *et al*. 2016; Jamy *et al*. 2016; Söderström *et al*. 2016) have reviewed the morphological classification of these taxa with the aim of improving the current taxonomic arrangement.

In spite of the various studies that have been conducted with *Plagiochila*, information about its spores is scarce (Erdtman 1965; Vojtkó 1993) or limited to brief comments in taxonomic descriptions (Grolle & Heinrichs 1999; Heinrichs & Gradstein 1999; Müller *et al*. 1999; Heinrichs *et al*. 2000; 2001; Heinrichs 2002; Hässel 2004; among others). Erdtman (1965) and Vojtkó (1993) presented a general description of spore surface and an evaluation of size, but with a small sample size. Descriptions of the spores of the genus in taxonomic studies are limited to superficial and sometimes inaccurate comments on ornamentation and size measurements.

Several characteristics of plants were crucial for their evolutionary transition to a terrestrial life, but the development of a durable and protective spore wall was essential (Brown & Lemmon 1988; Renzaglia *et al*. 2000; Wellman 2004; Wallace *et al*. 2011; Arteaga-Vazquez 2016). The spore — a single cell produced by meiosis — is the first stage of the gametophyte in the life cycle of liverworts (Brown & Lemmon 1988). Liverworts possess two layers of sporoderm, the special cell wall of the spore: an inner layer called the intine, which is composed of polysaccharides and is related to spore germination; and an external stratum called the exine, which composed of sporopollenin, a highly resistant polymer that provides resistance and protection (Olesen & Mogensen 1978; Neidhart 1979; Mogensen 1983; Blackmore & Barnes 1987; Brown & Lemmon 1988; Ito *et al*. 2007; Renzaglia *et al*. 2000; Wallace *et al*. 2011).

Many studies on spores have addressed different aspects of various groups (Heckman 1970; Blackmore & Barnes 1987; Brown & Lemmon 1980; 1984a; b;1988; 1991; Estébanez *et al*. 1997; Luizi-Ponzo & Barth 1998; 1999; Luizi-Ponzo & Melhem 2006; Caldeira *et al*. 2006; 2009; 2013; Yano & Luizi-Ponzo 2006; 2011; Rocha *et al*. 2008; Rodrigues & Luizi-Ponzo 2015; Savaroğlu 2015; Savaroğlu *et al*. 2017; Silva-e-Costa *et al*. 2017). However, little is known about morphology of the spores of the genus *Plagiochila*. In fact, it is worthy to note that sporophytes are infrequent among species of *Plagiochila* (Heinrichs 2002).

The present study aimed to perform a palynological evaluation of species of *Plagiochila* in order to: (1) analyze intra- and interspecific variation in spore size, (2) describe spore ornamentation and (3) sporoderm structure, and (4) determine whether infrageneric circumscriptions are supported by spore morphology.

Materials and methods

Sample selection and studied material

The research was developed using herborized botanical material loaned or donated by the following herbaria: Botanical Garden of Rio de Janeiro Herbarium (RB), Brazilian National Museum Herbarium (R), Herbarium Anchieta (PACA), Santa Cecília University Herbarium (HUSC), University of Kentucky Herbarium (KY), and Professor Leopoldo Krieger Herbarium (CESJ). Acronyms follow Thiers (2018).

As previously mentioned, the rarity of the occurrence of sexual reproduction in *Plagiochila* (Dumort.) Dumort. is remarkable, which makes finding sporophytes on these plants difficult (Schuster 1980; Heinrichs 2002; Gradstein & Costa 2003). Thus, 1000 specimens from the aforementioned herbaria of various species of *Plagiochila* were examined in search of specimens with sporophytes. All species for which a sporophyte was found, and which and enough material available for study, were analyzed, for a total of thirty-four specimens of seventeen species. The analyzed species, preceded by the name of their respective section, are: sect. *Arrectae* Carl - *Plagiochila bifaria* (Sw.) Lindenb; sect. *Fuscolutea* Carl – *P. fuscolutea* Taylor; sect. *Glaucescentes* Carl - *P. buchtiniana* Steph.; sect. *Hylacoetes* Carl - *P. macrostachya* Lindenb.; sect. *Plagiochila* – *P. asplenioides* (L.) Dumort. and *P. porelloides* (Tor ex. Ness) Lindenb.; sect. *Rutilantes* Carl - *P. gymnocalycina* (Lehm. & Lindenb.) Lindenb., *P. heteromalla* Lehm. & Lindenb., *P. rutilans* Lindenb., and *P. trichostoma* Gottsche.; and sect. *Vagae* Lindenb. - *P. corrugata* (Nees) Nees & Mont, *P. crispabilis* Lindenb., *P. disticha* (Lehm. & Lindenb.) Lindenb., *P. laetevirens* Lindenb., *P. patula* (Sw.) Lindenb., *P. raddiana* Lindenb., and *P. simplex* (Sw.) Lindenb. Species circumscription follows Gradstein (2015b) and the names are in accordance to the Tropicos Database (http://www.tropicos.org).

Light microscopy

For observation under light microscopy (LM), spores were prepared according to the method of Wodehouse (1935), for observation of cellular content, and by the acetolysis method proposed by Erdtman (1960). Both techniques were performed following the modifications recommended by Luizi-Ponzo & Melhem (2006). Spores were described using the terminology proposed by Punt *et al*. (2007) and the definitions of size classes of Erdtman (1952).

Scanning electron microscopy

For observation under scanning electron microscopy (SEM), capsules were fixed in 2.5 % glutaraldehyde for 24 hours and then washed in 0.05 M phosphate buffer solution. Post-fixation was performed with 2 % osmium tetroxide (OsO_4) in buffer solution for a period of two hours. The capsules were then dehydrated in an increasing ethanol series and dried in a critical point dryer (Silveira 2007). The capsules were opened under stereoscopic microscopy and the spores dispersed on stubs with double-sided carbon tape and covered with a 20 nm layer of gold. Non-fixed spores were also observed. The SEM analyses were undertaken at the Laboratório de Microscopia Eletrônica of the Universidade Federal de Juiz de Fora, the Centro de Microscopia of the Universidade Federal de Minas Gerais, and the Centro de Microscopia e Microanálise of the Universidade Federal de Viçosa.

Transmission electron microscopy

For observation under transmission electron microscopy (TEM), mature capsules were separated, fixed in 2.5 % glutaraldehyde for 24 hours, washed in 0.05 M phosphate buffer solution, and post fixed in 2 % osmium tetroxide in buffer solution. After dehydration in an increasing ethanol series, the material was embedded in Spurr resin and heated at 70 ºC for 48 hours. The material was cut in ultrathin sections (65-70 nm) and stained with uranyl acetate and lead citrate (Reynolds 1963). The TEM analyses were undertaken at the Centro de Microscopia of the Universidade Federal de Minas Gerais.

Statistical analyses

Spore size was assessed under light microscopy using acetolyzed material. When possible, based on the availability of palynological material, more than one specimen was examined for all species studied and with a reference specimen (RS) and comparison specimens (CS) being designated. For estimating largest diameter, 50 RS spores randomly chosen from three microscope slides were measured. When available, 30 CS spores randomly chosen from three microscope slides were analyzed. Descriptive statistics were calculated from the resulting data, including arithmetic mean (X), size range (X_{min}–X_{max}), standard deviation (S), standard error (Sx), coefficient of variation (CV% - obtained by the formula (S/X)*100) (Sokal & Rohlf 1995), 95 % confidence level (95 % CL), and 95 % confidence interval (95 %CI - X 95 %CL) (Sokal & Rohlf 1995) using Microsoft Excel (2016). Ten non-acetolyzed RS spores randomly chosen from three microscope slides were measured for sporoderm thickness and only the arithmetic mean calculated.

The measurements were not normally distributed (Shapiro-Wilk normality test, $p < 0.05$) and so the Kruskall-Wallis test, followed by the Dunnett's test (which are more appropriate tests for non-parametric data), were performed to test intra- and interspecific differences. Median values and data distribution were graphically evaluated. Statistical analysis and graphing were performed using R software v. 3.5.1 (R Development Core Team 2018) and JMP® 12 (SAS Institute, Cary, North Carolina, USA).

Cluster analysis

The degree of association among the studied species was evaluated by cluster analysis, using the unweighted pair-group average (UPGMA) algorithm and Jaccard similarity index, and calculating the cophenetic correlation coefficient, using the software Past 3.21 (Hammer *et al*. 2001).

Palynological, gametophytic and ecological data (Tab. 1) were organized in a qualitative binary matrix (Tab. 2). The palynological data included the following: spore size (< 26 μm or ≥ 26 μm), spore ornamentation (granules or long granules), and sporoderm thickness (two classes of sporoderm thickness were established using the formula h=A/k; where h is the class amplitude, A is the spore size amplitude, and k is the number of classes (Correa 2007). The gametophytic data included the following: branching type (*Frullania*-type or *Plagiochila*-type), androecia shape (single or fan-shaped), androecia position (terminal or intercalary), perianth base (naked or covered by bracts), and asexual reproduction (absent or present). The single ecological variable included was related to substratum: the species are reported as exclusive when occurring on only one type of substratum, and generalist when occurring on two or more types of substrata. The species *Plagiochila bifaria* was not included in this analysis due to a lack of information.

Results

The spores of the genus *Plagiochila* are monads that are isomorphic, apolar to weakly heteropolar, small to large in size (13.00 – 57.80 μm; Tab. 3, Figs. 1, 2), inaperturate and with a subcircular amb. Spore surface ornamentation is formed by granules, but the nature of these processes varies among the seventeen species studied (Figs. 1, 2), with four types being identified under LM and SEM: (1) regular and delicate granulate (RD), (2) irregular and coarse granulate (IC), (3) long granules with flattened apices (LF), and (4) long and straight granules (LS).

Ornamentation pattern I - RD consists of granules homogeneously distributed on the spore surface and having a regular shape (Fig. 1A - C). This pattern is possessed by *Plagiochila asplenioides* (Fig. 1A), *P. disticha* and *P. patula* (Fig. 1B, C). Spore size for these three species varies from small to medium (Tab. 3), and the sporoderm is thin (Tab. 4). Under LM this ornamentation appears as a blur in optical cut due to its small size and fine magnification (Fig. 1A, B), but under SEM the granules are easily identified (Fig. 1C).

Ornamentation pattern II - IC - comprises irregular shaped granules disorderly distributed on the spore surface (Fig. 1 D-H). This pattern is possessed by *P. gymnocalycina* (Fig. 1D), *Plagiochila heteromalla*, *P. laetevirens* (Fig. 1E), *P. porelloides* (Fig. 1F, G) and *P. raddiana* (Fig. 1H). Spore size varies from small to medium (Tab. 3), and the sporoderm is of variable thickness (Tab. 4). *Plagiochila gymnocalycina* has a more elaborate version of this pattern with overlapped and united granules (Fig. 1D), producing a gemmae-like appearance.

Ornamentation pattern III - LF – includes species whose spore surface is ornate with elongate and irregularly distributed granules that have flattened apexes (Fig. 2A-F). This pattern is possessed by *Plagiochila crispabilis* (Fig. 2A, C) and *P. simplex* (Fig. 2B, D-F). This type of granule is barely observable under LM (Fig. 2A), but can be clearly observed under SEM, including the flattened apex region (Fig. 2C-F). There is a region with smaller granules restricted to a particular area (Fig. 2D) that suggests it may have been the site of contact during the tetrad stage.

Table 1. Palynological, gametophytic and ecological aspects of the studied species of *Plagiochila* (Dumort.) Dumort.

Species	Spore Size	Spore Ornamentation	Sporoderm Thickness	Branching Type	Substratum Occupation	Androecia Shape	Androecia Position	Perianth Base	Asexual Reproduction	Section
P. asplenioides	Small	RD	1.5327	Frullania	Exclusive	Single	Intercalary	Bracts	Absent	*Plagiochila*
P. buchtiniana	Medium	LS	1.2636	Frullania	Exclusive	Single	Intercalary	Bracts	Absent	*Glaucescentes*
P. corrugata	Medium	LS	1.3923	Frullania	Exclusive	Single	Intercalary	Bracts	Absent	*Vagae*
P. crispabilis	Medium	LF	1.6497	Frullania	Generalist	Fan-Shaped	Terminal	Bracts	Propagules	*Vagae*
P. disticha	Medium	RD	1.2519	Frullania	Generalist	Single	Terminal, Intercalary	Bracts	Propagules	*Vagae*
P. fuscolutea	Large	LS	1.5444	Plagiochila	Generalist	Single	Intercalary	Bracts	Absent	*Fuscolutea*
P. gymnocalycina	Small	IC	2.0241	Plagiochila	Generalist	Single	Intercalary	Naked	Caducous leaves	*Rutilantes*
P. heteromalla	Medium	IC	1.4742	Plagiochila	Exclusive	Single	Terminal	Naked	Caducous leaves and fragments	*Rutilantes*
P. laetevirens	Medium	IC	1.3221	Frullania	Generalist	Sngle	Terminal, Intercalary	Bracts	Propagules	*Vagae*
P. macrostachya	Medium	LS	1.8369	Plagiochila	Generalist	Fan-Shaped	Terminal	Bracts	Caducous leaves	*Hylacoetes*
P. patula	Medium	RD	1.1700	Frullania	Generalist	Single	Intercalary	Bracts	Propagules	*Vagae*
P. porelloides	Small	IC	1.1457	Plagiochila	Exclusive	Single	Terminal, Intercalary	Bracts	Absent	*Plagiochila*
P. raddiana	Medium	IC	0.9594	Frullania	Generalist	Single	Terminal	Bracts	Propagules	*Vagae*
P. rutilans	Medium	LS	1.8837	Plagiochila	Generalist	Single	Intercalary	Bracts	Caducous leaves	*Rutilantes*
P. simplex	Small	LF	1.5561	Plagiochila	Generalist	Single	Intercalary	Naked	Absent	*Vagae*
P. trichostoma	Medium	LS	1.2168	Plagiochila	Generalist	Single	Intercalary	Bracts	Caducous leaves and fragments	*Rutilantes*

Table 2. Binary matrix showing the arrangement of palynological, gametophytic and ecological aspects of *Plagiochila* (Dumort.) Dumort. Spore size: <26 μm = 1, ≥ 26 μm = 0; spore ornamentation: types RD and IR = 0, types LF and LS = 1; sporoderm thickness: ≥ 1.4900 μm = 1; < 1.4900 μm = 0; branching: Plagiochila-type = 1; Frullania-type = 0; Substratum occupation: exclusive = 1, generalist = 0; androecia shape: fan-shaped = 1, single = 0; androecia position: intercalary = 1, terminal = 0; perianth base: bracts present = 1, naked = 0; asexual reproduction: present = 1, absent = 0.

Species	Size	Ornamentation	Sporoderm Thickness	Branching Type	Substrum Occupation	Androecia Shape	Androecia Position	Perianth Base	Asexual Reproduction
P. asplenioides	1	0	1	0	1	0	1	1	0
P. buchtiniana	0	1	0	0	1	0	1	1	0
P. corrugata	0	1	0	0	1	0	1	1	0
P. crispabilis	0	1	1	0	0	1	0	1	1
P. disticha	1	0	0	0	0	0	1	1	1
P. fuscolutea	0	1	1	1	0	0	1	1	0
P. gymnocalycina	1	0	1	1	0	0	1	0	1
P. heteromalla	0	0	0	1	1	0	0	0	1
P. laetevirens	0	0	0	0	0	0	1	1	1
P. macrostachya	0	1	1	1	0	1	0	1	1
P. patula	0	0	0	0	0	0	1	1	0
P. porelloides	1	0	0	1	1	0	1	1	0
P. raddiana	1	0	0	0	0	0	0	1	1
P. rutilans	0	1	1	1	0	0	1	1	1
P. simplex	1	1	1	1	0	0	1	0	0
P. trichostoma	1	1	0	1	0	0	1	1	1

Table 3. Morphometric data of acetolyzed spores of *Plagiochila*. Reference specimen indicated by an asterisk (n=50); comparison specimens, n=30.

Material	(X$_{min}$-X$_{max}$)	X ± S$_x$	S	95 % IC	CV (%)
Plagiochila asplenioides (L.) Dumort.					
Hanner, 5020 (MN) *	13.00 – 26.00	18.79 ± 0.36	2.60	18.06 – 19.52	13.84
Plagiochila bifaria (Sw.) Lindenb.					
M.C. Vaughan Bandeira (166934/RB)*	20.80 – 31.20	24.50 ± 0.32	2.28	23.85 – 25.15	9.30
Plagiochila buchtiniana Steph.					
J. Heinrichs *et al.*, 4128 (RB) *	41.60 – 52.00	46.54 ± 0.38	2.74	45.77 – 47.31	5.88
Plagiochila corrugata (Nees) Nees & Mont.					
L.A. Paiva, 320 (CESJ) *	24.70 – 36.40	29.22 ± 0.38	2.70	28.46 – 29.98	9.24
A. Sehnem, 2407 (PACA)	22.10 – 31.20	26.21 ± 0.42	2.34	25.37 – 27.05	8.92
A. Sehnem, 1103 (PACA)	19.50 – 39.00	27.84 ± 0.71	3.92	26.38 – 29.30	14.08
A. Sehnem, 4742 (PACA)	26.00 – 37.70	30.57 ± 0.58	3.20	29.38 – 31.76	10.46
Plagiochila crispabilis Lindenb.					
D.P. Costa *et al.*, 5100 (RB) *	23.40 – 33.80	27.89 ± 0.30	2.18	27.27 – 28.5	7.81
D.M. Vital, 4946 (RB)	23.40 – 36.40	28.21 ± 0.39	2.19	27.40 – 29.02	7.76
P. Occhioni, s/n (166931/ RB)	18.20 – 31.20	25.22 ± 0.59	3.27	24.00 – 26.44	12.96
Plagiochila disticha (Lehm. & Lindenb.) Lindenb.					
D.P. Costa *et al.*, 2895 (RB) *	15.60 – 23.40	20.18 ± 0.23	1.68	19.70 – 20.66	8.32
D.P. Costa *et al.*, 2769 (RB)	20.80 – 31.20	24.32 ± 0.39	2.11	23.52 – 25.12	8.67
D.P. Costa *et al.*, 2685 (RB)	20.80 – 28.60	25.93 ± 0.41	2.22	25.09 – 26.77	8.56
D.P. Costa *et al.*, 2615 (RB)	18.20 – 28.60	22.99 ± 0.44	2.41	22.09 – 23.89	10.48
D.P. Costa *et al.*, 2623 (RB)	31.20 – 20.80	25.87 ± 0.48	2.67	24.88 – 26.86	10.32
Plagiochila fuscolutea Taylor					
H. Anton & J. Heinrichs (373220/RB)	46.80 – 72.80	57.05 ± 0.89	6.35	55.25 – 58.85	11.13
J. Henrichs, 3915 (RB)	31.20 – 52.00	37.65 ± 0.92	4.99	35.76 – 39.54	13.25
Plagiochila gymnocalycina (Lehm. & Lindenb.) Lindenb.					
N.D. Santos *et al.*,840 (RB) *	18.20 – 31.20	23.46 ± 0.41	2.91	22.63 – 24.29	12.40
D.P.Costa, 3890 & S.R. Gradstein (RB)	19.50 – 27.30	23.27 ± 0.36	1.99	22.52 – 24.02	8.55
D.P.Costa, 3876 & S.R. Gradstein (RB)	15.60 – 23.40	19.34 ± 0.45	2.45	18.41 – 20.27	12.66
Plagiochila heteromalla Lehm. & Lindenb.					
N.D. Santos *et al.*, 475 (RB) *	26.00 – 39.00	30.94 ± 0.38	2.71	30.17 – 31.71	8.75
Plagiochila laetevirens Lindenb.					
M. Bandeira (223901/RB) *	20.80 – 33.80	26.17 ± 0.37	2.66	25.42 – 26.92	10.16
Plagiochila macrostachya Lindenb.					
E. T. Amorim, P2 (CESJ) *	21.45 – 36.40	26.87 ± 0.38	2.3	26.10 – 27.64	10.16
Plagiochila patula (Sw.) Lindenb.					
D.P. Costa *et al.*, 5054 (RB) *	18.20 – 41.60	28.16 ± 0.61	4.30	26.94 – 29.38	15.26
D.P. Costa *et al.*, 2885 (RB)	15.60 – 23.40	19.24 ± 0.48	2.63	18.26 – 20.22	13.66
Plagiochila porelloides (Torr. ex. Ness) Lindenb.					
D.N. McLetchie, s/n (UKY) *	14.30 – 23.40	19.43 ± 0.36	2.56	18.71 – 20.15	13.17
Plagiochila raddiana Lindenb.					
E.C. Rente, 426 (MN) *	10.40 – 18.20	13.80 ± 0.24	1.70	13.32 – 14.28	12.31
D.P. Costa *et al.*, 2659 (RB)	16.25 – 23.40	20.29 ± 0.40	2.17	19.47 – 21.11	10.69
D.P. Costa *et al.*, 5062 (RB)	20.80 – 31.20	26.06 ± 0.52	2.86	25.00 – 27.12	10.97
D.P. Costa *et al.*, 2596 (RB)	20.80 – 28.60	23.44 ± 0.37	2.06	22.68 – 24.20	8.78
Plagiochila rutilans Lindenb.					
M.C. Vaughan Bandeira (166936/RB) *	27.00 – 41.60	34.16 ± 0.48	3.45	33.18 – 35.14	10.09
D. Sucre 2453. & P.I.S. Braga (RB)	28.60 – 39.00	36.31 ± 0.52	3.23	35.11 – 37.51	8.89
Plagiochila simplex (Sw.) Lindenb.					
P. Dusén, 450 (MN) *	16.90 – 26.00	21.36 ± 0.27	1.95	20.81 – 21.91	9.12
D.P. Costa *et al.*, 4863 (RB)	15.60 – 28.60	22.44 ± 0.45	2.50	21.51 – 23.37	11.14
Plagiochila trichostoma Gottsche					
J. Heinrichs *et al.*, 4323 (RB)*	20.80 – 31.20	25.15 ± 0.38	2.69	24.39 – 25.91	10.69

Ornamentation pattern IV - LS – this ornamentation consists of an ornate spore surface with elongate granules that have a smooth and straight shape, resembling bacula (Fig. 2G-I). The species that possess this pattern are: *Plagiochila bifaria*, *P. buchtiniana*, *P. corrugata* (Fig. 2H), *P. fuscolutea*, *P. macrostachya*, *P. rutilans*, and *P. trichostoma* (Fig. 2G, I). A region with smaller granules, suggestive of the proximal pole, was observed in some species. Among this group, *P. corrugata* is notable for having endosporic and intracapsular germination (Fig. 2H).

Sporoderm thickness varied from 0.9 µm to 1.8 µm among the studied species (Tab. 4). Observations under TEM revealed the sporoderm to comprise one or two electron-translucent layer(s) corresponding to the intine (Figs. 1I, 2F – inner intine and outer intine when two layers are present), and two electron-dense layers compounded by lamellae deposition (Figs. 1I, 2F), corresponding to the exine divided into nexine and sexine. The sexine lamellae have a perpendicular to inclined arrangement (Figs. 1I, 2F). A stratified intine was
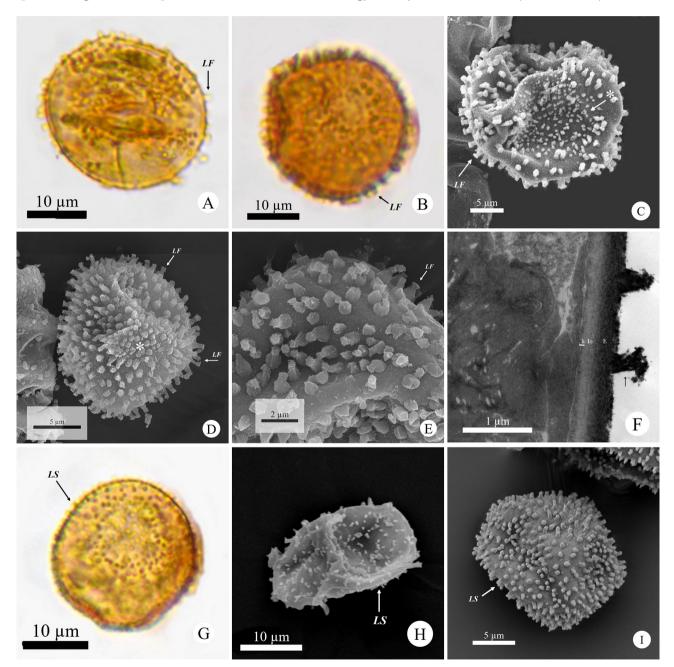

Figure 1. Photomicrographs and electromicrographs of spores of species of *Plagiochila* (Dumort.) Dumort. Ornamentation type I - **A.** *Plagiochila asplenioides*, LM; **B.** *P. patula*, LM; **C.** *P. patula*, SEM; Ornamentation type II – **D.** *P. gymnocalycina*, LM; **E.** *P. laetevirens*, LM; **F – G.** *P. porelloides*, SEM – arrow with asterisks = proximal face; **H.** *P. raddiana*, SEM; **I.** *P. disticha*, TEM – arrow = lamellar slips. RD = regular and delicate granule; IC = irregular and coarse granule. In I: In = inner intine, Io = outer intine, E = exine.

observed for the spores of *P. disticha* (Fig. 1I), including a granular inner layer, with a mix of electron-translucent and electron-dense components, that is in contact with cell contents, and an outer layer, with a great amount of electron-translucent elements, that is in contact with the nexine.

Descriptive statistical analyses showed that the species analyzed here differ significantly in spore size (Fig. 3). Furthermore, there was significant intraspecific variation in spore size (Fig. 4) for those species for which CS was available

(see Tab. 3), with the exception of *P. simplex* (Fig. 4I), which did not exhibit significant intraspecific variation in spore size.

The variability of spore size was confirmed by coefficients of variation (Tab. 3). The lowest values, around 8 %, were for *P. buchtiniana* and RS and CS1 in *P. cripabilis*. Values around 10 % were observed in a great number of species, while values greater than 12 % were detected in at least one specimen of *P. asplenioides*, *P. crispabilis*, *P. fuscolutea*, *P gymnocalycina*, *P. raddiana P. patula*, and *P. porelloides*.

Figure 2. Photomicrographs and electromicrographs of spores of species of *Plagiochila* (Dumort.) Dumort. Ornamentation type III – **A.** *Plagiochila crispabilis*, LM; **B.** *P. simplex*, LM; **C.** *P. crispabilis*, SEM – arrow with asterisks = proximal area; **D-E.** *P. simplex*, SEM –asterisks = proximal face; **F.** *P. simplex*, TEM; **G.** *P. trichostoma*, LM; **H.** *P. corrugata*, SEM; **I.** *P. trichostoma*, SEM. LF = long granule with flattened apex; LS = long and straight granule. In F: In = inner intine, Io = outer intine, E = exine, arrow = lamellar slips.

For almost all the studied species for which a CS could be analyzed, the mean value for the CS did not fit the CI established for the reference specimen (Tab. 3).

Cluster analysis revealed five groups with similarity above 0.5 (Fig. 5 - G1 to G5) and a cophenetic correlation coefficient of 0.8138. The group G1 assembles *P. disticha*, *P. laetevirens*, *P. patula*, and *P. raddiana*; group G2 group unites *P. asplenioides*, *P. buchtiniana*, P. *corrugata*, and *P. porelloides*; group G3 is the largest, being represented by *P. fuscolutea*, *P. gymnocalycina*, *P. rutilans*, *P. trichostoma*, and *P. simplex*; group G4 is formed by *P. crispabilis* and *P. macrostachya*; and group G5 is represented by the single species *P. heteromalla*. Referring to the binary matrix (Tab. 4),it is possible to unravel the characteristics shared among species of the same group. The species of G1 share ornamentation type (rounded granules), sporoderm thickness, *Frullania*-type branching, generalist substratum occupation, single androecia, perianth covered by bracts, and some type of asexual reproduction. The species of G2 also share a single androecia and a perianth covered by bracts, but do not possess a asexual reproduction structure, and are exclusive in substratum occupation. In addition, these species also share androecia positioned terminally, although and branching type is variable. The species of G3 have generalist substratum occupation, *Plagiochila*-type branching and single androecia; while the species of G4 have generalist substratum occupation, fan-shaped androecia, and perianth covered by bracts. The species of this last group also share some spore characteristics such as ornamentation and sporoderm thickness. The species *P. heteromalla* is the only species of group G5.

Table 4. Morphometric data for sporoderm thickness of spores of *Plagiochila* (n=10).

Species	Sporoderm thickness (µm)
P. asplenoides	1.5327
P. buchtiniana	1.2636
P. corrugata	1.3923
P. crispabilis	1.6497
P. disticha	1.2519
P. fuscolutea	1.5444
P. gymnocalycina	2.0241
P. heteromala	1.4742
P. laetevirens	1.3221
P. macrostachya	1.8369
P. patula	1.17
P. porelloides	1.1457
P. raddiana	0.9594
P. rutilans	1.8837
P. simplex	1.5561
P. trichostoma	1.2168

Discussion

Plagiochila is an important genus, being distributed worldwide and one of the most speciose genera of liverworts. Palynological information can lead to a better understanding of the taxonomy and ecology of the species of *Plagiochila*. The present study revealed the spores of this genus to be variable, especially with regard to spore size and sporoderm ornamentation, which were able to separate the studied species into four spore types.
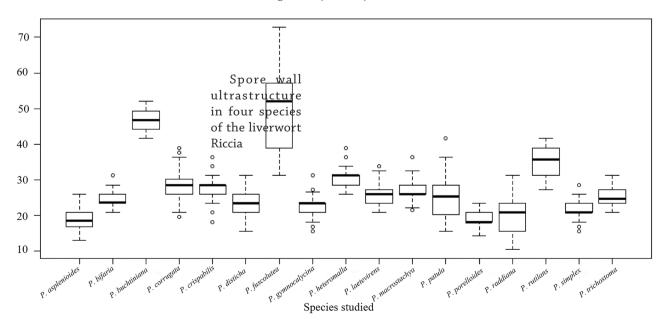
Plagiochila **(Dumort.) Dumort**

Figure 3. Boxplots representing the spore size distribution within *Plagiochila* (Dumort.) Dumort. Error bars above and below the box indicate the 90th and 10th percentiles, respectively, while white circles represent the outliers.
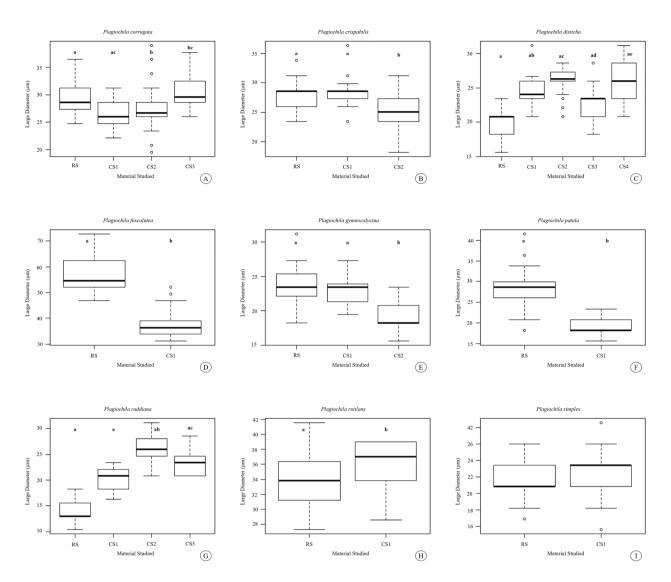
Figure 4. Boxplots representing the spore size distribution within those species for which RS and CS were observed. Error bars above and below the box indicate the 90th and 10th percentiles, respectively, while white circles represent the outliers. Different letters (a, b, c, d, e) represent statistical differences among treatments (Krukal-Wallis test, Dunn's post hoc test, p < 0.05).

Spore size in *Plagiochila*

Average spore size in *Plagiochila* varied from 13.80 μm to 57.05 μm, being classified as small to large (Erdtman 1952). Spore size is a variable characteristic among liverworts. For example, spores of *Chonecolea doelligeri* (Chonecoleaceae) measured 16.00 μm in polar view (Yano & Luizi-Ponzo 2006), while species belonging to Frullaniaceae (Zhao *et al.* 2011), Dumortieraceae (Yano & Luizi-Ponzo 2011), Porellaceae (Zhao *et al.* 2011), and Ricciaceae (Steinkamp & Doyle 1979; Zhao *et al.* 2011), varied widely from 31 μm (medium-sized) to 127 μm (giant).

Related to the present study, Erdtman (1965) reported that the spores of *P. asplenioides* have an average size of 15 μm, while Vojtkó (1993) reported the spores of *P. porelloides* to be 17.80 μm. Some taxonomic studies have included

notes about spores of species of *Plagiochila*, especially regarding size and surface ornamentation. Heinrichs & Gradstein (2000) described the spores of *P. disticha* as ranging in size from 16 μm to 28 μm (- 47 μm), and those of *P. raddiana* ranging 18 μm – 25 μm (- 45 μm); Heinrichs *et al.* (2000) reported a range of 33 μm – 54 μm for spores of *P. buchtiniana*; Heinrichs *et al.* (2001) reported a range of 23 μm – 28 μm for spores of *P. rutilans*; and Heinrichs *et al.* (2004a) reported 18 μm – 52 μm for *P. corrugata*. The present study found slightly narrower ranges of variation for the spores of these species.
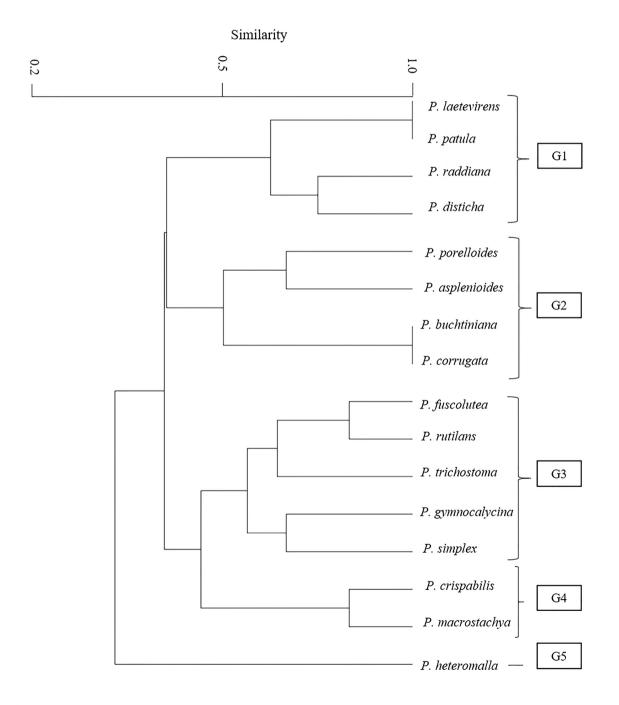
Lophocoleineae Schljakov, which includes Plagiochilaceae, possesses exosporic spore germination (Crandall-Stotler *et al.* 2009), but some studies (Heinrichs *et al.* 2000; 2004a; Heinrichs 2002) have reported spores of *Plagiochila* released with 1-8 cells. Endosporic germination

was also observed in the present study with the release of spores with 1-5 cells (and even intracapsular germination) in *P. corrugata*.
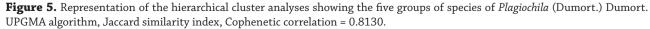
The coefficient of variation for spore size for the species studied here ranged from 8 % to 12 %. A coefficient of variation of around 10 % has been commonly found by palynological treatments of bryophytes, such as Luizi-Ponzo & Barth (1998; 1999), Luizi-Ponzo & Melhem (2006), Rocha *et al.* (2008), Caldeira *et al.* (2009; 2013), Yano & Luizi-Ponzo

(2011), and Rodrigues & Luizi-Ponzo (2015). Heinrichs (2002) stated that the average size of spores of *Plagiochila* vary considerably, while Heinrichs & Gradstein (1999) reported ca. 50 % variation in spore size for *Plagiochila longiramea*.

Sporoderm structure and surface ornamentation

Two layers of sporoderm, the intine and the exine, were observed in the species of the present study, which is typical

Figure 5. Representation of the hierarchical cluster analyses showing the five groups luf species of *Plagiochila* (Dumort.) Dumort. UPGMA algorithm, Jaccard similarity index, Cophenetic correlation = 0.8130.

of for bryophyte spores (Clarke 1979; Neidhart 1979; Mogensen 1983). Erdtman (1965) reported that spores of *P. asplenioides* have a very thin exine. The sporoderm of the species studied here is indeed quite thin, being no more than 2 μm thick and even thinner than 1 μm for some species.

Analyses made under TEM revealed that the exine is divided into the nexine, an inner layer, and the sexine, an outer layer. Similar exine configurations have been observed for spores of both mosses and liverworts (Heckman 1970; Steinkamp & Doyle 1979; Brown & Lemmon 1988; Estébanez *et al*. 1997; Yano & Luizi-Ponzo 2006; 2011; Rocha *et al*. 2008; Caldeira *et al*. 2013; Brown *et al*. 2015; Silva-e-Costa 2015; Rodrigues & Luizi-Ponzo 2015).

Lamellar deposition of exine elements in liverworts was first reported by Heckman (1970) and Brown & Lemmon (1988). The present study found perpendicular sexine elements in two species — *P. disticha* and *P. simplex*. Heckman (1970) reported similar sexine patterns in two other liverworts — *Lophocolea heterophylla* and *Chiloscyphus polyanthos*. This author described these elements that shape spore ornamentation as "lamellar slips". Both species studied by Heckman (1970) belong to the family Lophocoleaceae, which is, as is Plagiochilaceae, a member of Lophocoleineae.

Two light electron-dense layers were identified in *P. disticha*, which correspond to intine. The intine is the last layer to be formed and is directly related to spore germination (Neidhart 1979; Mogensen 1983; Brown & Lemmon 1988). Mogensen (1983) pointed out that the intine can have little stratification, as observed in the present study; Studying the spore wall structure in Jungermanniales, Heckman (1970) presented a similar configuration of a stratified intine in *L. heterophylla*; even though it was not mentioned in the text, the stratification is easy recognized in the image provided by the paper.

McClymont & Larson (1964) described a multistratified intine for *Archidium alternifoliuim*; Nilsson (1990) observed it in pollen grains of Apocynaceae; Estébanez *et al*. (1997) observed a one to three-layered intine in *Grimmia*; Luizi-Ponzo & Melhem (2006) described a stratified intine for mature spores of *Helicophyllum torquatum*; and, finally, Medina & Estébanez (2014) reported intine stratification for *Orthotrichum ibericum* and *Orthotrichum striatum*, and argued that intine stratification may be due to the environmental and developmental condition of spores.

Different dispositions of the exine element may be related to early contact at the proximal pole during spore wall formation. Brown & Lemmon (1991) observed that during wall formation of liverwort spores, especially in Jungermanniidae, the exine is generally thinner and less ornamented on the proximal face than in other areas of the spore wall, and might represent a region for germination.

Some authors have described some *Plagiochila* spores as ornate with bacula (Heinrichs & Gradstein 2000; Heinrichs *et al*. 2001; 2004a; 2005a; b; Heinrichs 2002),

verrucate, or even vermiculate (Inoue 1982; Muller *et al*. 1999; Heinrichs 2002; Groth *et al*. 2003; Heinrichs *et al*. 2004b). In a treatment of Plagiochilaceae of North America, Schuster (1980) described the ornamentation of the spores as "finely granulose". In a compilation of literature data, Heinrichs (2002) described two basic patterns of spore ornamentation for *Plagiochila*: (1) verrucate-vermiculate structures, and (2) baculate structures (varying from "bacula *sensu strictu*" to pila). Observing the images provided by these authors, in can be concluded that these ornamentation patterns are, actually, granules with different and variable shapes and morphologies. Punt *et al*. (2007) defined *bacula* as "a cylindrical, free standing exine element more than 1 μm in length and less than this in diameter". In a similar way, a *verruca* is an element more than 1 μm wide; and *vermiculate* is used to describe *rugulate* pollen and spores. Thus, none of these terms fit the spore ornamentation observed in *Plagiochila*. The present study encountered elements that were less than 1μm long and wide, and when they were larger than this the elements were clearly groups of united processes. Heinrichs (2002) stated that the spores of *Plagiochila* are, necessarily, trilete with a weakly developed laesure under SEM observation. Due to their thin and delicate spore wall, spores of species of *Plagiochila* are easily damaged during processing for SEM, thus giving the appearance of an irregular trilete mark. However, when spores are observed under different kinds of preparation, this affirmation is clearly denied. Observations of spores under LM (Figs. 1A, B, D, E; 2A, B, G) prior to acetolysis, affirm that they are not trilete, but indeed tremendously fragile and easily folded. This fragility is related to the thin sporoderm and, especially, the delicacy of the nexine, which becomes folded during processing for SEM (Figs. 1F, H; 2C, E, H). It is important to note, however, that in spite of this fragility, the sporoderm tolerates acetolysis due its sporopollenin content (Mogensen 1983; Brown & Lemmon 1988).

Interpretation of hierarchical clustering and the taxonomic implications

The hierarchical clustering analyses revealed five groups with a cophenetic correlation coefficient higher than 0.8, which represents low distortion and relatively reliable data (Rohlf & Fisher 1968). These five groups, G1 to G5, did not correspond to the current infrageneric classification of *Plagiochila*. The common characteristics shared by species of the same group, for groups G1 to G4, are mostly morphological characteristics of the gametophyte. Furthermore, species with the same ornamentation type described in this study were also separated by cluster analysis. To G1 and G4, indeed, spore ornamentation was a shared variable for species of group G1 and for species of group G4, while surface ornamentation varied

within groups G2 and G3. Although these groups did not correspond directly to either infrageneric circumscriptions or ornamentation types, the palynological information added to gametophyte morphology clearly formed new groups, suggesting that spore data may contribute to understanding the phylogenetic relationships within *Plagiochila*. Spore morphology is genetically based (Clarke 1979), and thus can be evolutionary information and useful in phylogenetic analyses.

Palynology can make an important contribution to taxonomic studies, especially when dealing with taxa that present taxonomic difficulties. The species of *Plagiochila* of the present study are quite homogeneous with regard to gametophyte characteristics, but they differ in spore structure, size, sporoderm strata, and surface ornamentation. As demonstrated here, different ornamentation types were observed and can useful in species descriptions.

Acknowledgements

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