



Spores of Radulaceae (Marchantiophyta) exhibit a level of micromorphological diversity far beyond expectation

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ABSTRACT

Radulaceae is one of the most isolated lineages of leafy liverworts. This family contains three genera and between 200 and 350 extant species worldwide. Most species belong to the genus *Radula*, which is subdivided into five subgenera and remains taxonomically challenging. In the framework of an integrative taxonomic revision of Radulaceae in tropical America, we are paying special attention to understudied features that may be taxonomically informative, such as spores. Here, we perform the first palynological evaluation of sixteen species of Radulaceae. The spores were processed by standard palynological techniques and described using light and electron microscopy. The spores of Radulaceae are isomorphic monads, apolar in species of the genus *Radula* and cryptopolar in *Cladoradula*, usually small to medium-sized, rarely large, inaperturate, with a circular to slightly elongated outline. The spore surface is ornamented with rounded elements, here called Granulate-type ornamentation, or with elongated elements, called Echinete-type ornamentation, and shows differences in each species. The palynological data, especially spore ornamentation, can make an important contribution to Radulaceae taxonomy for separating species or groups of species. The groups found here, however, do not fully correspond to generic and infrageneric circumscriptions as supported by molecular-phylogenetic evidence.

Keywords: *Cladoradula*, liverworts, palynology, *Radula*, SEM, taxonomy.

Introduction

Radulaceae Müll. Frib. (Porellales Schljakov suborder Radulinae R.M.Schust.) is one of the most isolated lineages of the leafy liverworts (Marchantiophyta Stotl. &

Crand.-Stotl. subclass Jungermanniidae Engl.) (Crandall-Stotler *et al.*, 2009). Distinctive morphological traits of the family include rhizoids in tufts on the lobule surface, *Radula*-type branches (originating from a stem epidermal cell and therefore associated with an unmodified leaf),

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very large, brown oil bodies, absence of underleaves, and tubular, dorsoventrally flattened perianths (Yamada, 1979; Schuster, 1980; Gradstein *et al.*, 2001; Oliveira-da-Silva *et al.*, 2021). The center of diversity is in the humid tropical to warm temperate regions, despite being distributed in all continents apart from Antarctica, and most species grow as epiphytes in moist tropical forests, from the lowlands to the upper montane belt (Gradstein *et al.*, 2001).

Until very recently, Radulaceae has been considered a monogeneric family with *Radula* Dumort. as its only genus. Recently, Renner *et al.* (2022) proposed two small additional genera based on molecular and morphological evidence, *Cladoradula* (Spruce) M.A.M. Renner *et al.*, with seven accepted species distributed throughout the Tropics and in eastern Asia and North America, and *Dactyloradula* (Devos *et al.*) M.A.M. Renner & Gradst. with only *D. brunnea* (Steph.) M.A.M. Renner & Gradst. in Japan, South Korea, Kuril Island, and British Columbia (Choi *et al.*, 2021); Renner *et al.*, (2022) erroneously cited *D. brunnea* as being endemic to Japan. The genus *Radula* alone contains between 200 and 350 species worldwide, included in five subgenera: *Amentuloradula* Devos *et al.*, *Metaradula* R.M. Schust., *Odontoradula* K. Yamada, *Radula*, and *Volutoradula* Devos *et al.* (Devos *et al.*, 2011; Renner *et al.*, 2022). The subgeneric classification is mostly based on molecular data (Devos *et al.*, 2011); the morphological definition of the subgenera remains problematic and the subgeneric assignment of a considerable number of species is still unclear.

An integrative taxonomic revision of Radulaceae in tropical America based on morphological and molecular evidence is under preparation by the first author. One of the specific objectives of this study is to pay attention to understudied features that potentially can be taxonomically informative, such as stem anatomy, oil bodies, cuticle ornamentation, asexual reproductive structures, presence/absence of stem perigynium, and characters of the sporophyte generation.

Sporophytes, although short-lived and rather rarely observed in *Radula*, produce spores which are the first cells of the gametophytic stage. Spores play a crucial role in dispersal (mainly by wind) and the establishment of new populations (van Zanten & Pócs, 1981; Medina & Estébanez, 2014; Glime, 2015). In systematics, spore features such as germination, ornamentation shape, and size, have allowed the identification of different bryophyte lineages (Bischler-Causse *et al.*, 2005; Goffinet & Shaw, 2009).

Various studies have reported data on the size and ornamentation of spores of *Radula* species from Africa, Asia, North America, and Macaronesia (Erdtman, 1957; 1965; Jones, 1977; Yamada, 1979; Schuster, 1980; Bouman & Dirkse, 1990). Jones (1977) was the first to propose the taxonomic usefulness of spore characteristics in *Radula* based on a study of the species from Africa. Schuster (1980) emphasized the general importance of sporophyte characters to unravel the taxonomy of *Radula*. One of the

first detailed descriptions of *Radula* spores was presented by Udar & Kumar (1983) in their treatment of the new species *Radula pandei* Udar & Kumar. The latter authors distinguished the spores of the new species from those of *R. tasmanica* Steph. utilizing scanning electron microscopy (SEM). SEM images were also used in the description of the spores of *Radula complanata* (L.) Dumort. and *R. tabularis* Steph. by Udar & Srivastava (1984).

In a survey of morphological characters of the sporophytes of 28 Australasian *Radula* species, Renner & Braggins (2005) recognized seven different spore types based on their size and ornamentation and emphasized the significance of spores in the taxonomy of Radulaceae. Further studies providing descriptions of *Radula* spores and highlighting their taxonomic significance include Renner (2006), Renner *et al.* (2010), Promma & Chantanaorrapint (2015), and Promma *et al.* (2018).

Information on Radulaceae spores in the literature is somewhat heterogeneous regarding the descriptive terminology, complicating taxonomic interpretation. Here we use a standardized palynological approach to (1) analyze variation in spore size and spore ornamentation, and (2) to determine whether generic and subgeneric circumscriptions are supported by spore morphology.

Material and Methods

Material

This study was based on herbarium material obtained on loan from the Federal University of Bahia (ALCB), University of Göttingen (GOET), Friedrich Schiller University Jena (JE), Missouri Botanical Garden (MO), New York Botanical Garden (NY) and Environmental Research Institute of São Paulo (SP). Mature sporophytes are rare in Radulaceae as well as in many other families of leafy liverworts (e.g. Silva-e-Costa & Luiz-Ponzo, 2019) and more than 1000 specimens were examined in search of sporophytes. In total, 25 specimens (16 species) with mature capsules in sufficient quantities were selected for palynological analysis. The following species, organized by genus and subgenus, were analysed:

Cladoradula (Spruce) M.A.M. Renner *et al.*: *C. boryana* (F. Weber) M.A.M. Renner *et al.*

Radula subg. *Amentuloradula* Devos *et al.*: *Radula flavifolia* (Hook.f. & Taylor) Gottsche *et al.*, and *R. ligula* Steph.

Radula subg. *Metaradula* R.M. Schust. (sect. *Epiphyllae* Grolle): *R. flaccida* Lindenb. & Gottsche, *R. mammosa* Spruce.

Radula subg. *Odontoradula* K. Yamada: *R. decora* Steph.

Radula subg. *Radula*: *R. javanica* Gottsche and *R. portoricensis* Steph.

Radula subg. *Volutoradula* Devos *et al.*: *R. episcia* Spruce, *R. involvens* Spruce, *R. pallens* (Sw.) Mont., *R. sinuata* Steph., *R. subinflata* Lindenb. & Gottsche, *R. venezuelensis* K.

Yamada, *R. voluta* Gottsche, Lindenb. & Nees, *R. xalapensis* Nees & Mont.

Light microscopy - LM

Permanent spore slides were prepared at the Palynology Laboratory of the Museu Paraense Emílio Goeldi (MPEG) and images were taken in the Microscopy Laboratory of MPEG using a light microscope Leica DM6 B with a camera.

For observation of cellular content under light microscopy, spores were prepared according to the method of Wodehouse (1935). For a better visualization of the surface of the sporoderm under LM, we performed the acetolysis method of Erdtman (1960), with modifications following Luiz-Ponzo & Melhem (2006). The acetolysis method is a standard palynological preparation, which removes the cellular content and the intine. It is widely used in palynology, both in studies dealing with pollen grains (e.g., Denk & Tekleva, 2006; Martins *et al.*, 2011; Matamoro-Vidal *et al.*, 2016; Mezzonato-Pires *et al.*, 2018; Subasinghe Arachchige *et al.*, 2022) and with spores of bryophytes (e.g., Luiz-Ponzo & Melhem, 2006; Savaroğlu & Erkara, 2008; Caldeira *et al.*, 2013; Luiz-Ponzo & Silva-e-Costa, 2019; Passarella & Luiz-Ponzo, 2022).

Spores were described using terminology and definitions of size classes proposed by Punt *et al.* (2007) and Erdtman (1952), respectively.

Scanning electron microscopy - SEM

Scanning electron microscopy (SEM) images were obtained at the Institutional SEM Laboratory of the MPEG, using a Tescan Mira 3 electron microscope with FEG (field emission gun) electron gun. For SEM preparation, spores were placed on aluminum supports using conductive carbon tape and coated with gold in an Argon atmosphere at a pressure of 2.10^{-2} mbar for 2':30". To obtain the images, a voltage acceleration of 5 kV and a working distance of 15 mm were used. Images of the spores were edited using the program Gimp 2.10.34.

Data analysis

Spores were measured under light microscopy using an eye objective with a millimeter ruler calibrated with a millimeter glass slide. Acetolysis was carried out on a single specimen per species (= reference specimen; RS). Additional specimens of the species, when available, served as comparison specimens (CS). To estimate the diameter of the spores, 50 spores of the RS and 30 of CS were chosen randomly from three to five microscope slides for measurement and analysis. Descriptive statistics was done using Microsoft Excel (2016) and included size range (X min–X max), median, skewness, kurtosis, and values of 25 percentile and 75 percentile.

A Kruskal-Wallis test was applied to test the intraspecific variance of the morphometric data of acetolyzed spores (Hollander & Wolfe, 1973), followed by the Wilcoxon test. Appropriate tests were applied for non-parametric data without normal distribution (Shapiro-Wilk test, $p < 0.05$) and homoscedasticity (Levene test, $p > 0.05$). Analysis and graphic presentation of the data were carried out with the R software (version 4.3.2, R Core Team, 2023).

To determine the similarity of the studied species, palynological, morphological, and ecological data were transformed into binary data (Table 1) and plotted in a matrix for hierarchical cluster analysis (Supplementary Material 1). The "eclust" function of the *factoextra* package (Kassambara & Mundt 2020) in R software (version 4.3.2, R Core Team, 2023) was used to perform k-means and h-clust clustering.

Results

Spores of Radulaceae are isomorphic monads, apolar in species of the genus *Radula* and, exceptionally, cryptopolar in *Cladoradula boryana*. They are usually small to medium-sized, 16.66–38.88 μm , rarely larger, 50.00–66.66 μm as in *Radula flavifolia*, inaperturate, with a circular to slightly elongated outline (Table 2, Figs. 1, 2, 3). The ornamentation of the spore surface includes two distinct patterns: I) ornamentation with rounded elements, here called Granulate-type ornamentation, and II) ornamentation with elongated elements, here called Echinete-type ornamentation.

The Granulate-type ornamentation consists of granular projections, which are very close to each other and evenly distributed across the spore surface. The granules may be rough, formed by the union of nanogranules (Figs. 2A, 2C-D, 2H-J, 2L-M, 2O-P, 3A, 3C-D, 3H-J, 3L-M, 3O-P), or smooth (Figs. 2G, 3G). Rough granules occurred in *Cladoradula boryana*, *Radula episcia*, *R. flaccida*, *R. javanica*, *R. ligula*, *R. mammosa*, *R. pallens*, *R. sinuata*, *R. subinflata*, *R. voluta*, and *R. xalapensis*, whereas *R. javanica* had smooth granules.

Cladoradula boryana stands out by the occurrence of a small circular region of smaller granules on the spore surface (Figs. 1A, 2A). As this region is not associated with an aperture, the spores of *C. boryana* are characterized as being cryptopolar.

The spore ornamentation of *Radula episcia*, *R. mammosa*, *R. pallens*, and *R. subinflata* is very delicate, presenting a psilate appearance under light microscopy (Fig. 1C, I, J, M). However, under scanning electron microscopy, the nanogranules are regularly distributed and overlapping in *R. episcia*. *Radula pallens* and *R. mammosa* have spores with isolated nanogranules across the spore surface, and in *R. subinflata* occurs a fusion of nanogranules.

In *Radula sinuata* and *R. voluta*, the spore surface is formed by a continuous layer of partially fused nanogranules, presenting perforations (Figs. 2L, 2O, 3L, 3O). *Radula flaccida* is distinguished by nanogranules irregularly overlapping



Table 1. Information on palynology, gametophyte morphology, and ecological aspects adopted to perform cluster analysis.

Species	Type of spore	Spore Size	Spore Ornamentation	Type of granules	Apex of spine ornate	Heterogeneous ornamentation	Substrate preference	Stem sub-epidermis	Gynoeceal innovation	Asexual Reproduction	Auricle at lobule	Lobule insert	Leaf border	Trigones at leaf cells
<i>Cladoradula boryana</i>	Cryptopolar	Medium	Granulate	Granules with nanogranules	Absent	Present	Generalist	Present	Absent	Absent	Present	Transverse to oblique	Absent	Present
<i>Radula decora</i>	Apolar	Medium	Spine	Spine smooth	Present	Absent	Exclusive	Absent	Present	Absent	Absent	Longitudinal	Absent	Present
<i>Radula episcia</i>	Apolar	Small	Granulate	Granules with nanogranules	Absent	Absent	Generalist	Absent	Present	Absent	Absent	Longitudinal	Absent	Absent
<i>Radula flaccida</i>	Apolar	Small	Granulate	Granules with nanogranules	Absent	Absent	Exclusive	Absent	Present	Present	Absent	Longitudinal	Absent	Absent
<i>Radula flavifolia</i>	Apolar	Large	Spine	Spine smooth	Present	Absent	Generalist	Absent	Present	Absent	Absent	Longitudinal	Absent	Present
<i>Radula involvens</i>	Apolar	Medium	Spine	Spine with nanogranules	Absent	Absent	Exclusive	Absent	Present	Absent	Absent	Longitudinal	Absent	Present
<i>Radula javanica</i>	Apolar	Medium	Granulate	Granules smooth	Absent	Absent	Generalist	Absent	Present	Present	Absent	Longitudinal	Absent	Absent
<i>Radula ligula</i>	Apolar	Small	Granulate	Granules with nanogranules	Absent	Absent	Generalist	Absent	Present	Absent	Absent	Longitudinal	Present	Absent
<i>Radula mammosa</i>	Apolar	Small	Granulate	Granules with nanogranules	Absent	Absent	Exclusive	Absent	Present	Absent	Absent	Longitudinal	Absent	Absent
<i>Radula pallens</i>	Apolar	Small	Granulate	Granules with nanogranules	Absent	Absent	Generalist	Absent	Present	Present	Absent	Longitudinal	Absent	Absent
<i>Radula portoricensis</i>	Apolar	Medium	Spine	Spine smooth	Absent	Absent	Exclusive	Absent	Absent	Present	Absent	Longitudinal	Absent	Present
<i>Radula sinuata</i>	Apolar	Medium	Granulate	Granules with nanogranules	Absent	Absent	Generalist	Absent	Present	Present	Present	Longitudinal	Absent	Absent
<i>Radula subinflata</i>	Apolar	Medium	Granulate	Granules with nanogranules	Absent	Absent	Generalist	Absent	Present	Absent	Absent	Longitudinal	Absent	Absent
<i>Radula venezuelensis</i>	Apolar	Medium	Spine	Spine with nanogranules	Absent	Absent	Exclusive	Absent	Present	Present	Absent	Longitudinal	Absent	Present
<i>Radula voluta</i>	Apolar	Medium	Granulate	Granules with nanogranules	Absent	Absent	Generalist	Absent	Present	Absent	Present	Longitudinal	Absent	Present
<i>Radula xalapensis</i>	Apolar	Small	Granulate	Granules with nanogranules	Absent	Absent	Generalist	Absent	Present	Absent	Absent	Longitudinal	Absent	Absent



Table 2. Morphometric data of acetolyzed and non-acetolyzed spores of Radulaceae. Reference acetolyzed specimen indicated by an asterisk (n=50); comparison specimens (n=30).

Material	($X_{\min}$ – $X_{\max}$)	Median	Skewness	Kurtosis	25 percentile	75 percentile
<i>Cladoradula boryana</i> (F.Weber) M.A.M.Renner <i>et. al.</i>						
Dauphin 1631 (MO) *	27.77 – 33.33	29.62	0.30	-1.14	27.77	31.48
<i>Radula decora</i> Steph.						
Züendorf 21137 (JE) *	31.48 – 37.03	33.33	0.69	-1.07	31.48	37.03
<i>Radula episcia</i> Spruce						
Dauphin 2117 (MO) *	20.37 – 24.07	22.22	0	-1.21	20.37	24.07
Topanta & Quishpe 669 (MO)	18.51 – 24.07	19.44	0.96	-0.60	18.51	20.83
<i>Radula flaccida</i> Lindenb. & Gottsche						
Caldwell & Baker 15423 (MO) *	20.37 – 25.92	22.22	0.21	-1.32	20.37	24.07
Pursell 11732 (MO)	16.66 – 20.37	18.51	-0.33	-1.49	16.66	20.37
<i>Radula flavifolia</i> (Hook.f. & Taylor) Gottsche <i>et al.</i>						
Züendorf 21341 (JE) *	40.74 – 66.66	55.55	-0.35	0.71	51.85	59.25
Züendorf 21139 (JE)	42.59 – 50.00	46.29	0.07	-1.21	43.98	50.00
<i>Radula involvens</i> Spruce						
Topanta & Caranqui 1263 (MO) *	29.62 – 33.33	31.48	-0.19	-1.35	29.62	33.33
<i>Radula javanica</i> Gottsche						
Gómez 6577 (MO) *	29.62 – 35.18	31.48	0.50	-0.98	29.62	33.33
Montfoort & Ek 1117 (GOET)	25.92 – 29.62	27.77	-0.11	-1.09	27.31	29.62
<i>Radula ligula</i> Steph.						
Bastos 4311 (ALCB) *	20.37 – 24.07	22.22	0.17	-1.23	20.37	22.68
<i>Radula mammosa</i> Spruce						
Underwood 946a (NY) *	22.22 – 25.92	24.07	0.25	-1.31	22.22	24.53
<i>Radula pallens</i> (Sw.) Mont.						
Arroyo <i>et al.</i> 2590 (MO) *	20.37 – 29.62	24.07	1.02	0.88	24.07	25.92
Held & van Rhijn HH76 (GOET)	18.51 – 24.07	21.30	0.01	-1.39	18.51	22.68
Smith 10251 (MO)	18.51 – 24.07	20.37	0.10	-1.52	18.51	24.07
<i>Radula portoricensis</i> Steph.						
Breedlove 68131 (MO) *	25.92 – 31.48	27.77	0.33	-0.98	25.92	29.62
<i>Radula sinuata</i> Steph.						



Table 2. Cont.

Material	($X_{\min}$ – $X_{\max}$)	Median	Skewness	Kurtosis	25 percentile	75 percentile
Yano & Peralta 28562 (SP) *	27.77 – 33.33	29.62	0.22	-1.40	27.77	31.48
<i>Radula subinflata</i> Lindenb. & Gottsche						
Allen 11916 (MO) *	22.22 – 27.77	25.00	-0.01	-1.29	24.07	27.77
Bastos 5251 (ALCB)	24.07 – 27.77	25.92	0.06	-1.23	24.07	27.77
<i>Radula venezuelensis</i> K.Yamada						
Maxon & Harvey 8455 (JE) *	31.48 – 37.03	37.03	-0.70	-1.33	33.33	37.03
<i>Radula voluta</i> Gottsche et al.						
Gradstein et al. 6852 (GOET) *	33.33 – 38.88	37.03	-0.24	-1.13	33.33	37.03
Herzog s.n. (JE)	24.07 – 33.33	27.77	0.42	-0.76	27.31	30.55
Schäfer-Verwimp et al. 24270 (MO)	29.62 – 33.33	29.62	1.11	-0.82	29.62	33.33
<i>Radula xalapensis</i> Nees & Mont.						
Marko Lewis 881742A (MO) *	22.22 – 25.92	24.07	-0.42	-1.26	24.07	25.92

other nanogranules on the spore surface. *Radula ligula* can be separated from other species by the scarcity of nanogranules over the spore surface whereas *R. xalapensis* differs in the large amount of nanogranules overlapping on the spore surface (Figs. 3H, 3P). In *R. javanica*, the spore surface is scabrate and the granules are smooth.

The Echinete-type ornamentation consists of blunt spiny projections, which may be distant or very close to each other, evenly or unevenly distributed across the spore surface. The spines may be smooth, distant to each other, and evenly distributed, as in *Radula decora*, *R. flavifolia*, and *R. portoricensis* (Figs. 2B, 2E, 2K, 3B, 3E, 3K), or rough, covered by nanogranules, very close to each other, and unevenly distributed, as in *Radula involvens* and *R. venezuelensis* (Figs. 2F, 2N, 3F, 3N).

Among the species with smooth spines, *Radula decora* and *R. flavifolia* have, in addition, elongated elements with ornamented apices. *Radula decora* has flattened apices, while *R. flavifolia* has barbed apices. In addition, the two species can be separated by the spore surface being covered by nanogranules in *R. decora* and by anastomosing nanogranules, presenting perforations, in *R. flavifolia*. *Radula portoricensis* differs by spore surface with striated exine and ornamentation elements heterogeneous in size and shape, some similar to verrucae, but predominantly elongate, resembling spines.

Spore sizes in the species showed significant differences (chi-squared = 171.46, df = 19, $p < 2.2e-16$). The spores of *R. flavifolia* were significantly larger than those of the remaining species (Supplementary Material 2, Fig. 4) and were classified here as being “large”, with 75% of the spores varying between 51.85 to 59.25 μm in diameter (Fig. 4). *Radula portoricensis*, with spores classified here as “medium-sized”, along with those of eight other species (*C. boryana*, *R. decora*, *R. involvens*, *R. javanica*, *R. sinuata*, *R. subinflata*, *R. venezuelensis*, and *R. voluta*), had 75% of the spores ranging from 25.92 to 29.62 μm in diameter. In the remaining species, significant differences in spore size were not observed, with spore sizes varying mainly between 22.22 to 27.77 μm in *Radula mammosa*, *R. pallens*, *R. subinflata*, and *R. xalapensis*, as well as *R. episcia*, *R. flaccida* and *R. ligula*, and between 20.37 to 24.07 μm in *R. episcia*, *R. flaccida* and *R. ligula*.

The clustering of the species by the Kmean method revealed two groups with high intra-group similarity (Fig. 5): a first group (G1 in red) of species with Echinete-type spore ornamentation (pattern II), and a second group (G2 in blue) with Granulate-type spore ornamentation (pattern I).



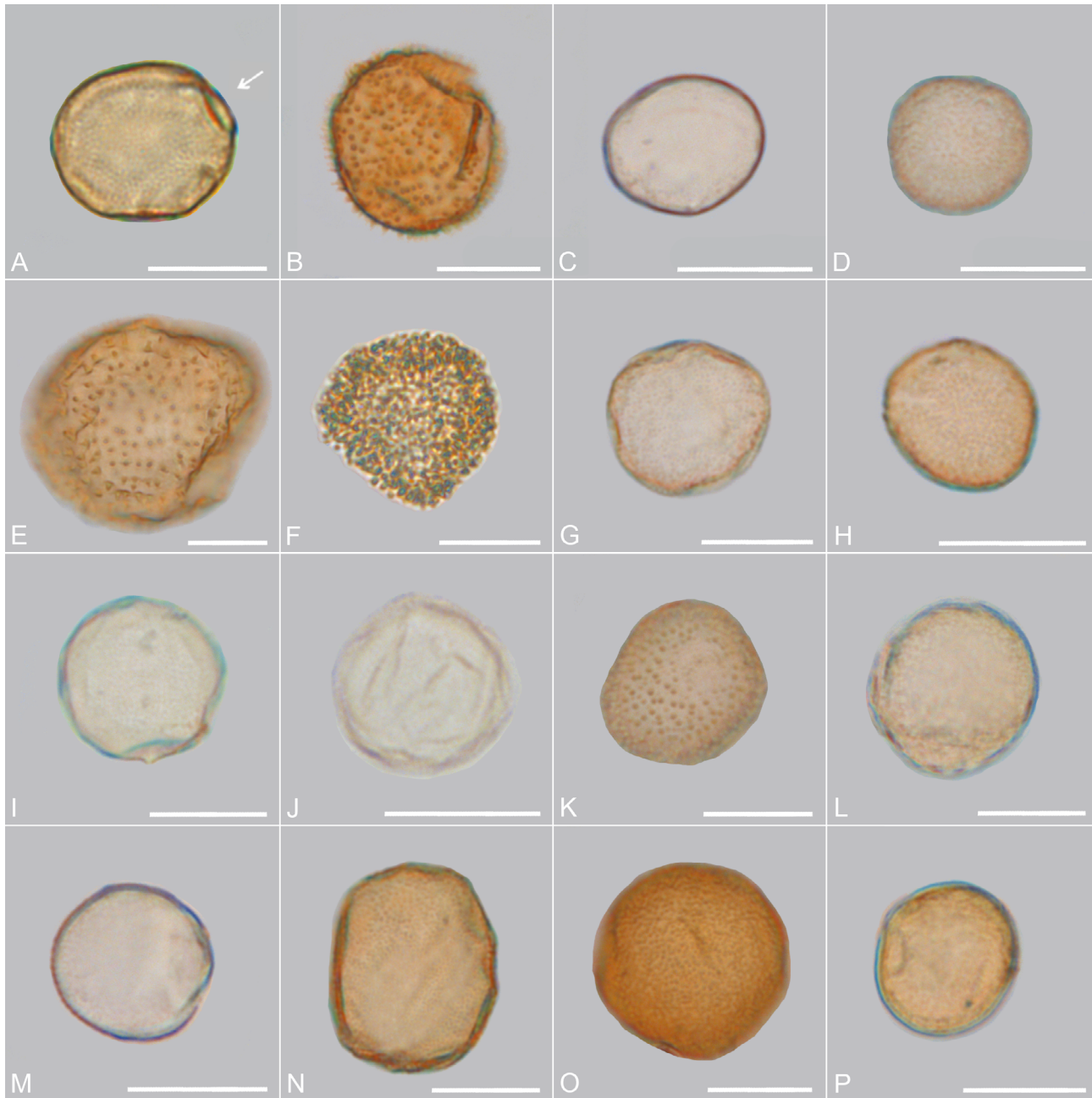


Figure 1. Photomicrographs of spores of species of Radulaceae under LM. **A.** *Cladoradula boryana*. **B.** *Radula decora*. **C.** *Radula episcia*. **D.** *R. flaccida*. **E.** *R. flavifolia*. **F.** *R. involvens*. **G.** *R. javanica*. **H.** *R. ligula*. **I.** *R. mammosa*. **J.** *R. pallens*. **K.** *R. portoricensis*. **L.** *R. sinuata*. **M.** *R. subinflata*. **N.** *R. venezuelensis*. **O.** *R. voluta*. **P.** *R. xalapensis*. Scale bar: 20 μ m.



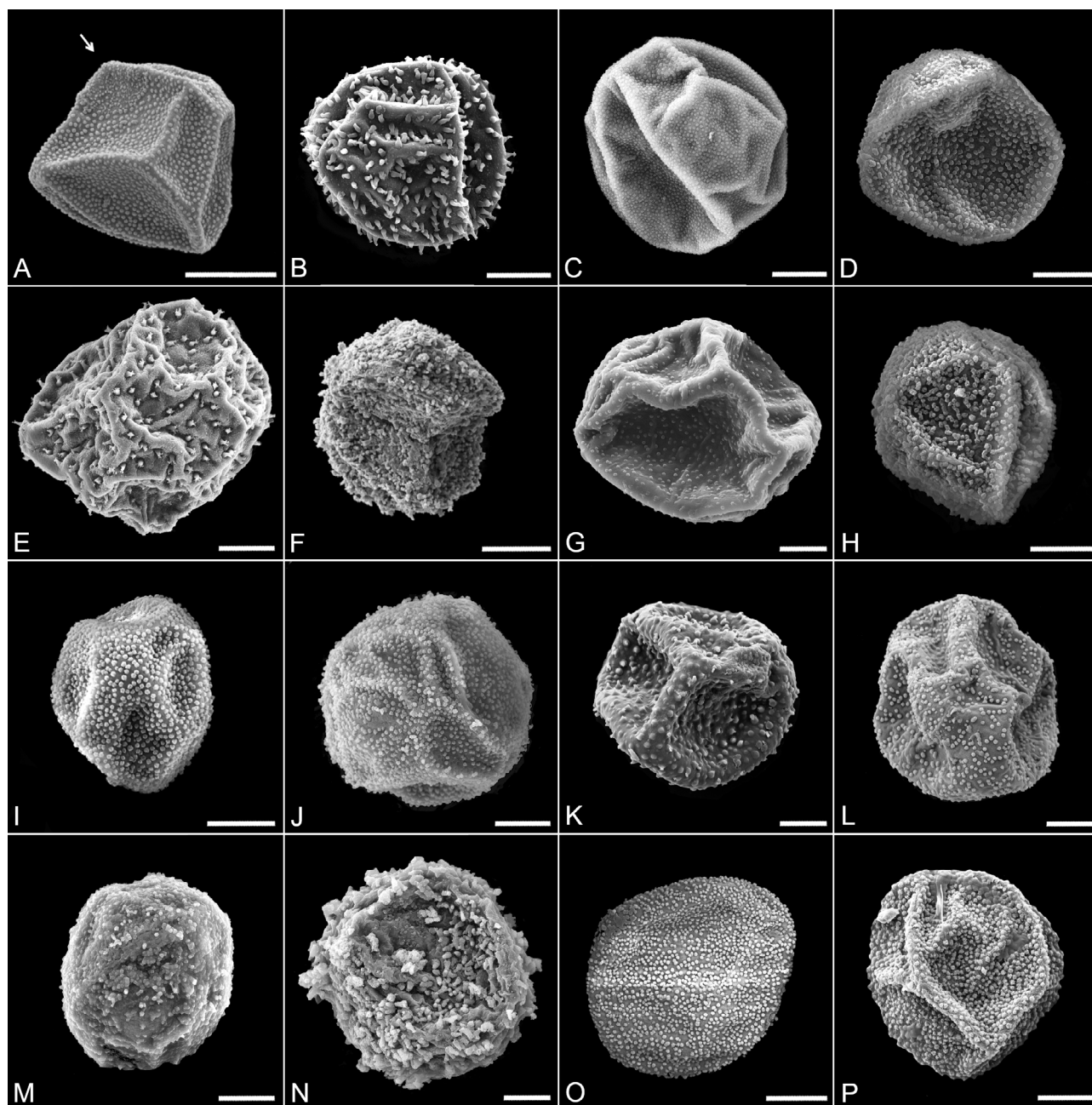


Figure 2. Electromicrographs of spores of species of Radulaceae under SEM. **A.** *Cladoradula boryana*. **B.** *Radula decora*. **C.** *R. episcia*. **D.** *R. flaccida*. **E.** *R. flavifolia*. **F.** *R. involvens*. **G.** *R. javanica*. **H.** *R. ligula*. **I.** *R. mammosa*. **J.** *R. pallens*. **K.** *R. portoricensis*. **L.** *R. sinuata*. **M.** *R. subinflata*. **N.** *R. venezuelensis*. **O.** *R. voluta*. **P.** *R. xalapensis*. Scale bar: A = 20 μm ; C-D, G-N, P = 5 μm ; B, E-F, O = 10 μm .

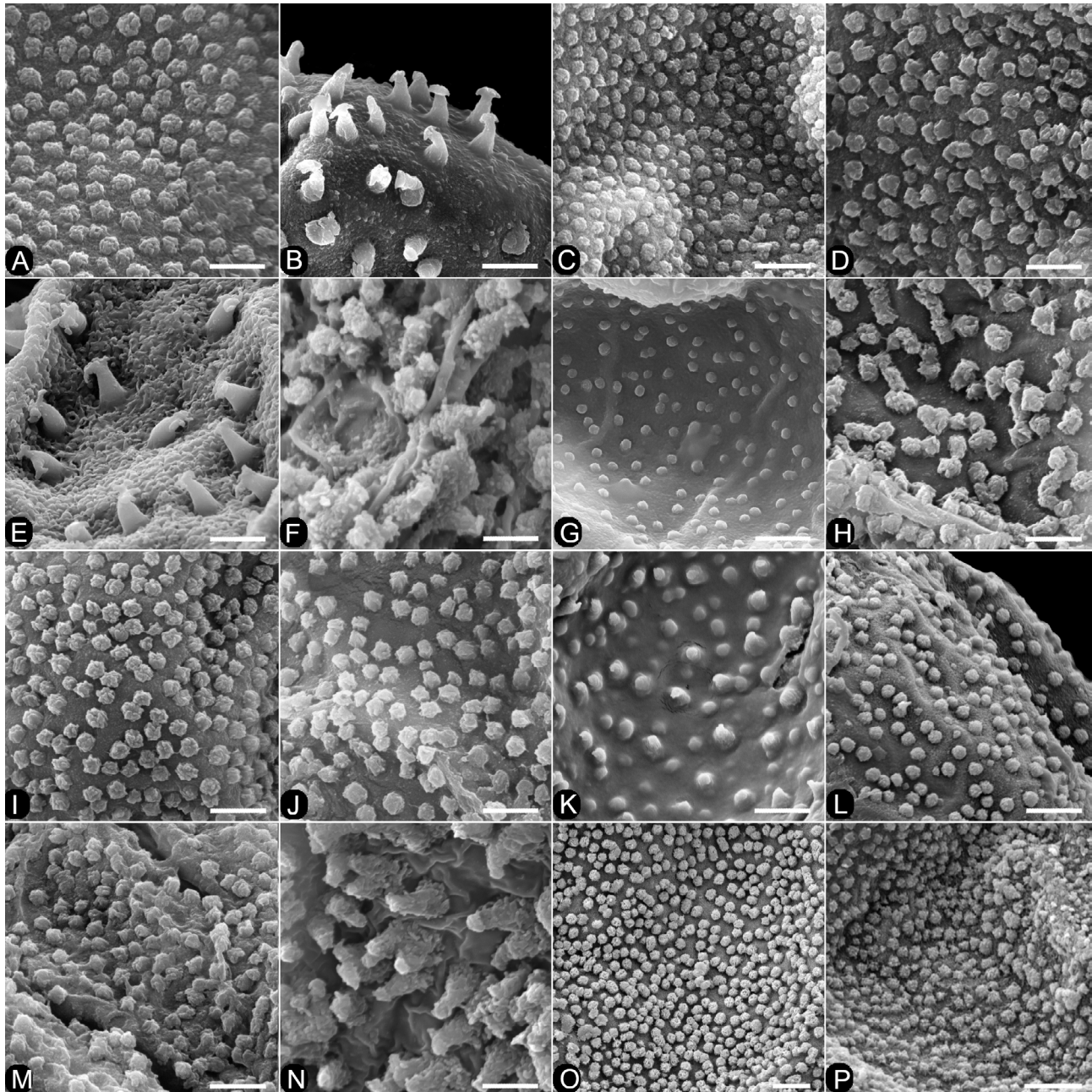


Figure 3. Electromicrographs of spore's ornamentation of species of Radulaceae under SEM. **A.** *Cladoradula boryana*. **B.** *Radula decora*. **C.** *R. episcia*. **D.** *R. flaccida*. **E.** *R. flavifolia*. **F.** *R. involvens*. **G.** *R. javanica*. **H.** *R. ligula*. **I.** *R. mammosa*. **J.** *R. pallens*. **K.** *R. portoricensis*. **L.** *R. sinuata*. **M.** *R. subinflata*. **N.** *R. venezuelensis*. **O.** *R. voluta*. **P.** *R. xalapensis*. Scale bar: A, C, D, F, H, I, J, M, N= 1 μ m; B, E, G, K, L, O, P= 2 μ m.



Discussion

This is the first study on spores of Radulaceae using the standard palynological approach, unraveling polarity and presence or absence of apertures, and applying the standard palynological terminology for spore size classes and ornamentation patterns, including granule and spine morphology.

The results confirm the need to use LM as well as SEM for adequate palynological description (Luizi-Ponzo & Melhem, 2006; Halbritter *et al.*, 2018). LM observation of acetolyzed and non-acetolyzed spores allows an accurate description of general characteristics such as spore size and outline, apolar or cryptopolar condition, presence or absence of an aperture, and, to some extent, spore ornamentation (Silva-e-Costa & Luiz-Ponzo, 2019; Passarella & Luiz-Ponzo, 2022). Description of ornamentation, however, is greatly enhanced with SEM, which allows the observation of nanogranules, details of the exine, and ornamentation of the elements.

Renner & Braggins (2005) investigated Radulaceae spores with SEM and using the terminology of Boros *et al.* (1993), adopting the term “echiane” instead of “bacula”. The authors named different types of spores by using species epithets (*e.g.*, *uvifera*-type). In the present study, however, spore types are named using morphological terms, with recognition of two main ornamentation types, the Granulate-type and the

Echinate-type (see Fig. 5). Additionally, we describe the variation observed within the two ornamentation patterns. We believe that the use of morphological terms for the naming of spore types enhances their application to other taxa (*e.g.*, families).

Thus far, spores of about 34 Radulaceae species have been studied (Erdtman, 1957; 1965; Heckman, 1970; Jones, 1977; Schuster, 1980; Udar & Kumar, 1983; Udar & Srivastava, 1984; Bouman & Dirkse, 1990; Renner & Braggins, 2005; Renner, 2006; Renner *et al.*, 2010; Promma & Chantanaorrapint, 2015; Promma *et al.*, 2018). This number is small, given that Radulaceae has more than 200 species worldwide (Renner *et al.*, 2022). In this study, spores of 16 species are comprehensively described for the first time, including 13 of which spores were previously unknown.

As pointed out by Renner & Braggins (2005), spores of Radulaceae show high micromorphological diversity, especially in ornamentation and size. Our findings lead to identifying polarity as a striking character separating the genera *Cladoradula* and *Radula* in the Neotropics and recognizing two patterns based on the spore ornamentation (Granulate-type and Echinate-type).

Cryptopolar spores were exclusively observed in the single neotropical species of *Cladoradula*, *C. boyana*, whereas apolar spores occurred in all species of *Radula* included in this study. *Cladoradula* was recently raised to the generic level by Renner *et al.* (2022) based on molecular data

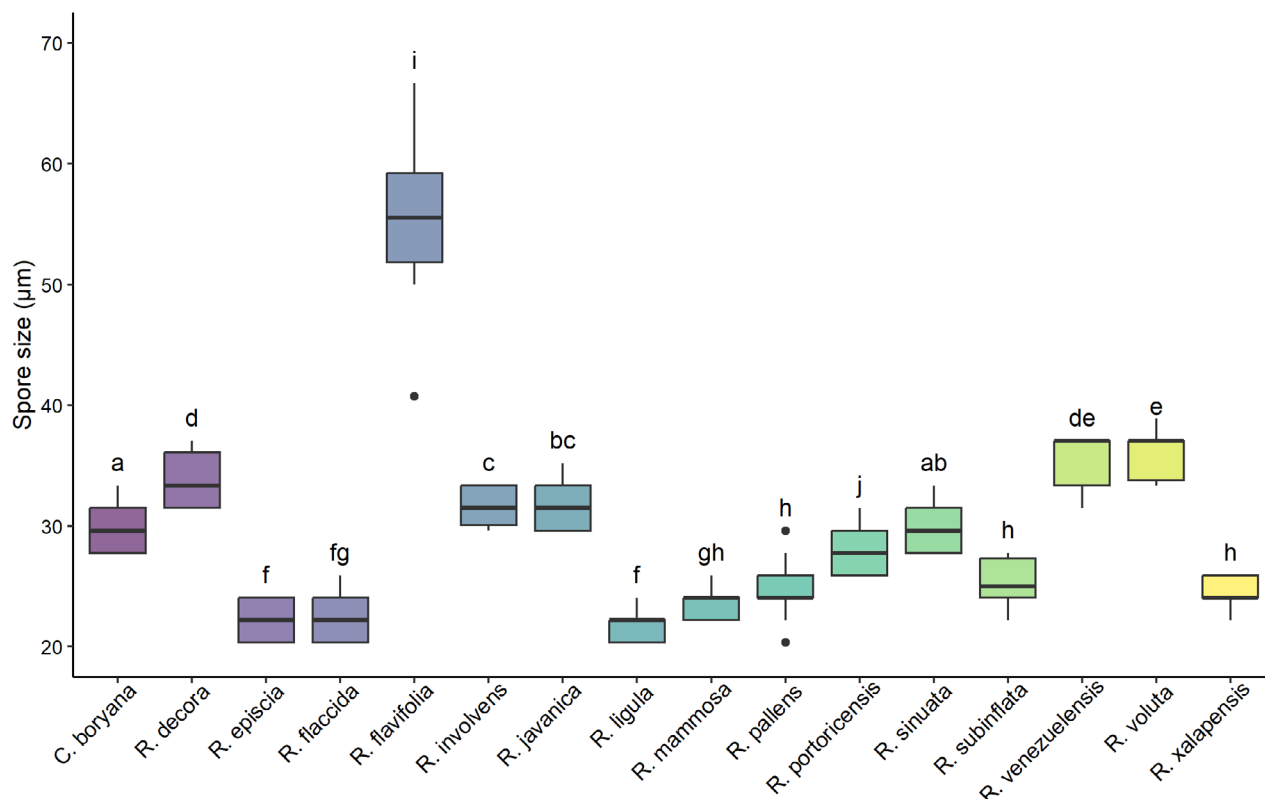


Figure 4. Boxplots representing the spore size distribution within Radulaceae. Letters above the boxplots (a-j) indicate similarity ($p > 0.05$; identical letters) or significant difference ($p < 0.05$; different letters) in interspecific morphometric variation of Radulaceae spores.

Cluster Dendrogram

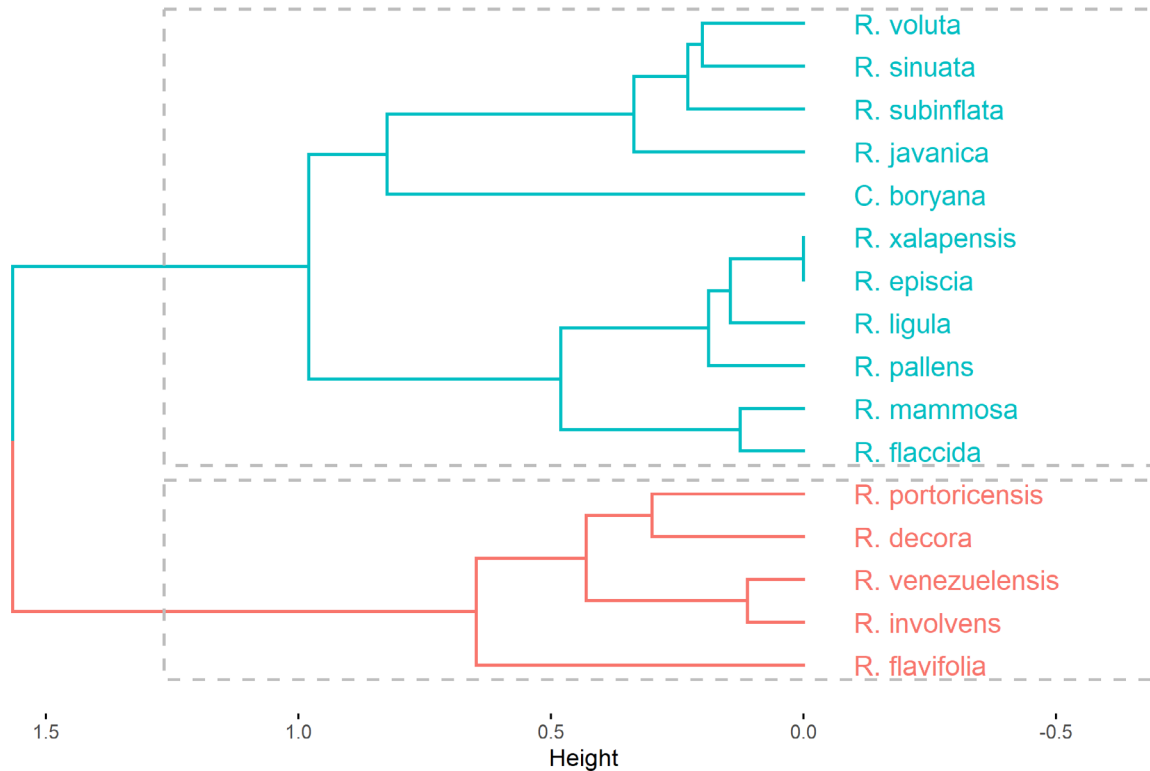


Figure 5. Detailed hierarchical clustering dendrogram of palynological, morphological, and ecological data for Radulaceae species.

and morphology, viz. (1) stems possessing a 1-3-layered subepidermis, (2) lobule insertion transverse to oblique, (3) gynoecea without innovation, and (4) capsules ovoid. The cryptopolar spores of *C. boryana* might be a further character separating *Cladoradula* from *Radula*. Spores of other *Cladoradula* species should be studied to verify their status as a diagnostic generic character.

Spore size in Radulaceae

In Radulaceae, spore size varies from 16.66 to 66.66 μm . According to Erdtman (1952), its classification ranges from small to large. The spore size of species appears to be independent of the size of the gametophyte. Thus, *Radula xalapensis* with plants measuring 2.5–4 mm wide and 5–8 cm long has smaller spores than *R. flavifolia* (1–1.2 mm wide and ca. 2 cm long) and similar in size to those of *R. mammosa* (1–2 mm wide and ca. 1 cm long).

Spores smaller than our findings were recorded for an African specimen of *Radula flaccida* (Jones, 1977; at least 15 μm), for the Asiatic *R. grandilobula* Promma & Chantanaorr. (Promma & Chantanaorr, 2015; at least 15 μm) and for the New Zealand *R. strangulata* Hook.f. & Taylor (as *R. silvosa* E.A.Hodgs. & Allison, in Renner & Braggins, 2005; 11–12 μm).

Radula flavifolia, so far, has spores with the largest diameter values recorded for Radulaceae, reaching 66.66

μm . Large spores have also been recorded for *R. aquilegia* (Hook.f. & Taylor) Gottsche *et al.* (44–50 μm) and *R. tabularis* Steph. (45–65 μm) (Jones, 1977; Udar & Kumar, 1983; Bouman & Dirkse, 1990).

Jones (1977) also reported spore sizes of *Cladoradula boryana* (as *R. boryana* (F.Weber) Mont.; 30–35 μm), *R. flaccida* (15–18 μm) and *R. voluta* (as *R. stipatiflora* Steph.; 22–27 μm). These measurements partially correspond to our findings for these species (see Table 2).

Renner & Braggins (2005) recognized three classes of spore size: spores smaller than 20 μm , between 20 and 30 μm (one species only), and larger than 30 μm in diameter. Our findings, however, reveal spores within two of these classes (e.g. *Cladoradula boryana*: 27.77–33.33 μm ; *Radula flaccida*: 16.66–25.92 μm).

Spore ornamentation

Despite using other terminologies, published descriptions of spore ornamentation in Radulaceae present the two main types of ornamentation observed in this study, even with particularities (i.e., sub-categories). Few studies investigated spores using SEM techniques (e.g., Renner & Braggins, 2005; Promma *et al.*, 2018). The latter technique allows a much more detailed view of the spore surface. The difference between SEM and light microscope views is easily seen in Figs. 1-3.



Spores ornamented by small, rounded granules formed by the union of nanogranules were described as tuberculate or “with tuberculate projections” by Renner & Braggins (2005). According to the latter authors, this type of ornamentation is found in members of at least three subgenera of *Radula*, including subg. *Amentuloradula* (*R. allamanoi* Gola, *R. buccinifera* (Hook.f. & Taylor) Gottsche et al., *R. marginata* Gottsche et al.), subg. *Metaradula* (*R. strangulata*) and subg. *Odontoradula* (*R. allisonii* Castle). In the present study, this ornamentation type was found in species of subg. *Volutoradula* and *Cladoradula*, thus being shared by old, basal lineages of Radulaceae (Patiño et al., 2017; Renner et al., 2022).

Udar and Krivastava (1984) and Promma et al. (2018) reported spores with “papillate” ornamentation in *Radula complanata* and *R. deflexilobula*, respectively, but SEM pictures (Udar & Krivastava, 1984: fig. 9; Promma et al., 2018: fig. 13-14) show smooth granules, similar to those found in *Radula javanica* in the present study. Interestingly, these three species are members of the same subgenus (subg. *Radula*). Further investigations on spore ornamentation of the species of subg. *Radula* should be carried out.

Spine projections barbed at apices in *Radula flavifolia* are striking similar in the spores of *R. physoloba* Mont. (subg. *Amentuloradula*) and *R. uvifera* (Hook.f. & Taylor) Gottsche et al., reported by Renner & Braggins (2005). Spores ornamented by elongated elements with flattened apices found in the present study for *R. decora*, finally, are not yet known from other *Radula* species but do occur in *Porella elegantula* (Mont.) E.A.Hodgs. (Renner & Braggins, 2005, plate 10, fig. 58).

Spore ornamentation and habitat

Renner & Braggins (2005) found a partial correlation between spore ornamentation and habitats occupied by the species. In our findings, species with spores ornamented by elongated elements (Echinate-type) are typically corticolous, corroborating the observations of the latter authors. The echinate projections may assist in spore adherence to the surface of twigs and stems (Engel & Gradstein, 2003). In the present study, we also observed that spores ornamented by rounded elements (Granulate-type), on the other hand, occur in species with a broader habitat preference, including the bark of trees, dead wood, living leaves, rock, and soil. Thus, *R. episcia* and *R. ligula* preferably grow on trees and wet rock while *R. flaccida* and *R. mammosa* are typically epiphyllous, growing on living leaves. Interestingly, the latter two species share a similar spore ornamentation with the epiphyllous *Radula buccinifera* from Australasia (Renner & Braggins, 2005).

Palynological contributions

Palynological analysis can make an important contribution to taxonomic studies of Radulaceae. Spores provide valuable characters and high micromorphological diversity for separating species and groups of species. These groups, however, do not fully correspond to the generic and infrageneric circumscriptions in Radulaceae as supported by molecular evidence (e.g., Devos et al., 2011; Patiño et al., 2017). Spores in Radulaceae can be taxonomically valuable when dealing with closely similar taxa, or with taxa that are difficult to define. Cryptopolar spores found in a member of *Cladoradula* might be a new diagnostic generic feature in Radulaceae. Palynological information from a larger number of species might contribute to a clearer understanding of phylogenetic relationships within Radulaceae.

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Authors' Contributions

FROS performed conceptualization, data curation, investigation, and writing - original draft. APLP contributed with palynological expertise, data curation, investigation, and writing - original draft. TTGT carried out statistical analysis. SRG and ALIB contributed to conceptualization, writing – review & editing.

Conflict of Interest

This paper has no conflict of interest.

Supplementary Materials

The following online material is available for this article:

Supplementary material 1. Binary matrix based on the information adopted to perform cluster analysis.

Supplementary material 2. Significance value according to the Wilcoxon test for interspecific morphometric variation

of Radulaceae spores. Pink indicates a significant difference ($p < 0.05$) and green a non-significant difference ($p > 0.05$).

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