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Palynological analysis of the genus *Ctenitis* (C. Chr.) C. Chr. (Dryopteridaceae) in the Southern Cone of America

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Abstract: The spore morphology and wall ultrastructure of 12 species of *Ctenitis* from Southern Cone of America were studied using light microscope, scanning and transmission electron microscope. The study was carried out with herbarium material from Argentine and Brazilian institutions. Equatorial diameters, polar diameters and laesura length were measured. The spores are monolete with echinate or folded ornamentation. In the echinate type, the spines are conical, with broad base and attenuate apex. In the rugate type, the folds are inflated, linear, sinuous, subglobose or handle-shape. The perispore surface is scabrate, rugulate, microverrucose or psilate. Stratification and ultrastructure in the species analyzed are very similar. The exospore is smooth and two-layered in section. Simple and branched channels are observed mainly in the outer exospore. The perispore is composed of two layers, the inner one forms the ornamentation and the outer covers all the outer and inner surfaces. Immature spores were found in all samples of *C. fenestralis*. The characteristics of the studied spores like macro-ornamentation, color and fold length provide relevant information to differentiate some species or groups of species within the genus.

Key words: fern, morphology, Southern Cone of America, spore, ultrastructure.

INTRODUCTION

The Dryopteridaceae family includes three subfamily, 26 genera and an estimated 2115 species (PPG I 2016). It has a cosmopolitan distribution and its center of diversity is in Southeast Asia (Zhang et al. 2013). *Ctenitis* (C. Chr.) C. Chr. is the fourth genus in terms of species richness within the family with 125 species of pantropical distribution and grows in middle and lower elevations of rainforest (PPG I 2016). The genus is characterized by the presence of catenate trichomes (ctenitoid hairs) on the adaxial surfaces of petioles, rachises and coastae and by the attenuated and thin termination of the veins (Viveros & Salino 2015, Viveros & Salino 2017). According to Viveros et al. (2018) the genus has 23 species in South America mostly in Brazil. For the Southern Cone of South America, 15 species of the genus *Ctenitis* are cited (Zuloaga et al. 2019, Flora do Brasil 2020): *C. abyssi* (Sehnem) Salino & Morais, *C. ampla* (Humb. & Bonpl. Ex Willd.) Ching, *C. anniesii* (Rosenst.) Copel., *C. aspidioides* (Presl.) Copel., *C. bigarellae* Schwartsb., Labiak & Salino, *C. deflexa* (Kaulf.) Copel., *C. distans* (Brack.) Ching, *C. eriocaulis* (Fée) Alston, *C. falciculata* (Raddi) Ching, *C. fenestralis* (C. Chr.) Copel., *C. flexuosa* (Fée) Copel., *C. laetevirens* (Rosenst.) Salino & Morais, *C. nervata* (Fée) R. S. Viveros & Salino, *C. paranaensis* (C. Chr.) Lellinger, and *C. submarginalis* (Langsd. & Fisch.) Ching.

The American members of *Ctenitis* were cataloged into three informal groups of species by Christensen (1913, 1920), based on morphological characters such as degree of blade division, indument, and rhizome type. Thus, the 1-pinnate-pinnatifid group with non-bullated scales ("*C. submarginalis*" group) includes most of the species with more representatives in South America, including among them *C. distans*, *C. eriocaulis*, *C. falciculata* and *C. submarginalis*. Other important group which has more representatives in Central America and West Indies to Bolivia and Northwestern Argentina, corresponds to the 2-4-pinnate-pinnatifid species with non-bullated scales ("*C. ampla*" group), where *C. ampla* is included. The third group ("*C. hirta*" (Sw.) Ching), 1-4-pinnate-pinnatifid with bullet scales, are mostly endemic to the Central America and West Indies (Hennequin et al. 2017).

For cytological studies, very few contributions of this nature have been carried out. Most of the species of the genus that have been studied are diploid or tetraploid (x= 41). As stated by Walker (1966, 1973) for Jamaica, Jarrett et al. (1968) for the Galapagos Islands, Bir (1996) for India and Tryon & Tryon (1982) for New Zealand, Sri Lanka and Japan.

As contributions of palynological studies of American species of *Ctenitis*, Tryon & Tryon (1982) affirm that the spores of the tropical species of America have two basic forms: echinated or folded. Tryon & Lugardon (1991) described and illustrated with Scanning Electron Microscope (SEM) the spores of 22 species of *Ctenitis* from America (Brazil, Costa Rica, Cuba, Colombia, Jamaica, El Salvador, Peru, Mexico), Southeast Asia (China, Japan, Tahiti), the Pacific Islands (New Guinea, Fiji, Hawaii) and the Reunion Islands in East Africa. When they analyzed general size, shape, surface and structure defined that the American spores of the genus are of two forms. Those spores belonging to the *C. submarginalis* group usually have short, inflated folds, while those of the *C. ampla* group are echinated. Only *C. ampla* and *C. submarginalis* from the Southern Cone species are briefly described by this authors with SEM.

Reed (1952) mentions that the *C. ampla* spores in North America has an echinate perispore. For this part, Moy (1988) illustrated with SEM the spores of this species from Venezuela, mentioning dimensions and some characteristics of the exospore and perispore. Furthermore, the author described *C. submarginalis* spores.

On the other hand, Moy (1988) and Tryon & Lugardon (1991) are the only authors who analyzed the spores of *Ctenitis* with Transmission Electron Microscope (TEM). Moy (1988) studied the spores of *C. ampla* from Venezuela and Tryon & Lugardon (1991) only analyzed the spores of *C. strigilosa* from Mexico.

The last contributions on the spores of the genus *Ctenitis* were made by Lebrão et al. (2014) who described the spores of *C. aspidioides*, *C. distans* and *C. falciculata* with Light Microscope (LM) for central Brazil.

On the other hand, the spores of some species of *Ctenitis* were described and illustrated in floristic or systematic works, based on observations with LM or SEM. The most of this information is scattered and scarce, mainly in South America.

While among the South American works, we can cite Viveros & Salino (2015) who described new species and made new combinations for *Ctenitis*, where the spores of five species of the genus, including *C. bigarellae*, *C. falciculata* and *C. paranaensis* were described and illustrated with SEM. Viveros et al. (2018) in the taxonomic monograph of the genus *Ctenitis* for South America, described and illustrated with SEM the spores of 24 taxa of the genus, stating that they are of two types, echinated and coarse folded and tuberculated.

The spores of the species growing in Southern Cone of South America have lightly been studied with LM and SEM, besides have not been studied with TEM yet. Furthermore, the sporoderm demands for a thorough study in order to define its ultrastructure and complexity. The aim of this work was to analyze the morphology and ultrastructure of the spores of the genus *Ctenitis* in Southern Cone of South America with LM, SEM and TEM.

MATERIALS AND METHODS

The study focused on the species cited for the Southern Cone of South America (Zuloaga et al. 2019), which includes the entire territories of Argentina, Chile, Uruguay, Paraguay and the 3 southern states of Brazil (Paraná, Rio Grande do Sul and Santa Catarina).

The study was done with herbarium material from the following institutions: BA, BHCB, CTES, LP, RB and SI (Thiers 2021). The spores were studied with LM, SEM and TEM. After LM and SEM analysis, *C. eriocaulis* and *C. submarginalis* were selected as representative for the study with TEM, considering that the available material was adequate and sufficient.

For the analysis with LM, the material was not subjected to any previous treatment. This is due to abrasive processes such as acetolysis (Erdtman 1960) can generate distortions like rupture of the perispore and increase in size, especially in spores with hollow folds like in the close genus *Dryopteris* (Nayar & Devi 1964, Prada 1990). For the study with SEM, the spores without treatment were placed into stubs with adhesive double-faced tape and coated with gold. For the TEM analysis, the material was treated following the technique of Rowley & Nilsson (1972): it was rehydrated with 0.1 M buffer and with 1% Alcian Blue (AB) for 2 hours; then the material was fixed with 1% glutaraldehyde + 1% AB in phosphate buffer for 12 hours; then it was washed for 15 minutes in phosphate buffer and postfixed with 1% Osmium tetraoxide in water + AB for 2 hours. The spores were dehydrated in an acetone series (30-100%) and subsequently embedded in a mixture of Spurr resin. The semi-thin sections 3 µm thick were stained with Toluidine blue and observed with LM. The ultrathin sections were stained with Uranyl acetate for 15 minutes followed by Lead citrate for 3 minutes.

The observations were made with a Zeiss EM 109T with Gatan ES1000W digital camera from the Instituto de Biologia Celular, Facultad de Medicina, Universidad de Buenos Aires, a JEOL JSMT-100 SEM from the Museo de Ciencias Naturales de La Plata and a Nikon E200 from Cátedra de Morfología Vegetal, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata. The following characteristics were analyzed: shape, equatorial and polar diameters, laesura, ornamentation and ultrastructure. For the description of the spores, the terms proposed by Tryon & Lugardon (1991) were used. The measures of spores were randomly estimated on 20 spores in each sample.

Studied material

C. ampla: Argentina, Salta, San Martín, Tartagal, Quebrada Naranjito, 15/07/1944, Schulz 5228 (LP).

C. anniesii: Brasil, Rio de Janeiro, Itatiaia, Parque Nacional de Itatiaia, 21/03/1942, Barros 707 (RB); Brasil, Rio de Janeiro, Itatiaia, Nova Picada Maromba, 21/03/1942, Brade 237 (RB); Brasil, Paraná, Tunas do Paraná, Colonia Joao XXIII, 2002, Ribas and Abe 4718 (SI); Brasil, Paraná, Villa Nova, 1905, Rosenstock 89 (SI).

C. aspidioides: Brasil, Rio de Janeiro, Santa Maria Madalena, Serra da Grama, 24/11/1977, Mautone 450 (RB).

C. bigarellae: Brasil, Minas Gerais, Catas Altas, RPPN Santuário do Caraça, Path to Pico da

Conceição, 26/08/2008, Viveros, Salino, Oliveira and Giacomim 22 (BHCB); Brasil, Minas Gerais, Parque Natural do Caraça Gruta do Padre Caio, Catas Altas, 2000, Salino 5958 (BHCB).

C. deflexa: Brasil, Rio de Janeiro, Nova Friburgo, California, 19/11/1922, Kuhlmann 110 (RB).

C. distans: Brasil, São Paulo, Iporanga, Apiaí, Parque Estadual Turístico do Alto Ribeira (PETAR), Núcleo Caboclos, 22/08/2012, Mazziero and Engels 1168 (RB).

C. eriocaulis: Brasil, Minas Gerais, Marliéria, Parque Estadual do Rio Doce, Ponte Queimada road, in front of Campolina, 29/05/2001, Stehmann, Sposito, Mota and Barros 2972 (BHCB); Brasil, Alagoas, São José da Lage, Usina Serra Grande, Mata Maria Maior, Grota do Gereba 2001, Pietrobom 5333 (BHCB).

C. falciculata: Brasil, Espírito Santo, Jatiboca, 13/05/1946, Brade, Pereira and Duarte 18187 (RB); Brasil, Santa Catarina, Armação do Sul, 15/12/1947, Senhem 3156 (RB); Brasil, Santa Catarina, Araranguá, Meleira, 1/2/1944, Reitz 1091 (RB); Brasil, Paraná, Villa Nova, Rio Negro, 1905, Rosenstock 79 (SI).

C. fenestralis: Brasil, São Paulo, Ubatuba, Parque Estadual da Serra do Mar, Núcleo de Picinguaba, Pico do Cuscuzeiro trail, close to the border between Rio de Janeiro and São Paulo, 2001, Salino, Dittrich, Morais and Meinberg 7285 (BHCB); Brasil, São Paulo, Ubatuba, Parque Estadual da Serra do Mar, Núcleo Picinguaba, Trilha da Cachoeira dos Macacos, 2001, Salino, Dittrich, Morais, Carvalho, Teixeira and Oliveira 7735 (BHCB); Brasil, Santa Catarina, Caçador, Pinheiral, 3-15 km W de Caçador, 1957¸ Smith and Klein 10894 (SI).

C. laetevirens: Brasil, Santa Catarina, Garuva, terrestrial herb; inside the fragment in an inclined place, 2009, Dreveck and Carneiro 1075 (BHCB); Brasil, Santa Catarina, 2010, near Serra da Santa before Morro do Funil, Pouso Redondo, Salino 14724 (BHCB).

C. nervata: Brasil, Paraná, Morretes, Estaçao Marumbi, 02/01/1986, Kummrow and Cordeiro 2701 (CTES); Brasil, Santa Catarina, Biguaçu, Fechinal, 18/01/1945, Reitz C1004 (RB); Brasil, Santa Catarina, Joinville, Entrada Dona Francisca, 1906, Rosenstock 118a (SI).

C. paranaensis: Brasil, Santa Catarina, Meleiro, Graranguá, 13/10/1943, Reitz 11 (RB).

C. submarginalis: Argentina, Tucumán, Tafí del Valle, 09/1919, Venturi 113 (BA); Argentina, Misiones, Santa Ana, 17/11/1913, Rodríguez 124 (BA); Argentina, Misiones, Guaraní, Predio Guaraní: 26°54'59' S- 54°12'18' O tramo II, 25/11/1993, Tressens, Cristobal, Khel et al. 4741 (LP); Argentina, Corrientes, Santo Tomé, Estancia Garruchos, 07/02/1972, Kaprovickas, Critobal, Maruñak et al. 21319 (LP); Argentina, Buenos Aires, Ensenada, Reserva Natural Punta Lara, 2006, Ramos Giacosa 24 (LP); Brasil, Rio Grande do Sul, Canguçú, 20/06/1968, Ceroni 4948 (CTES); Brasil, Paraná, Toledo, 15/11/1963, Pereira and Hatschbach 30248 (LP); Brasil, Paraná, Foz do Iguaçu, P. N. Iguaçu, Río Iguaçu, 25º37'36 -54°28'54', 2006, Labiak, Matos, Boeger and Muschner 3802 (SI); Brasil, Rio Grande do Sul, Rolante, Casacata do Chuvisquiero, 1988, Diesel 70004 (SI); Paraguay, Canindeyú, Jeju-mi, path Jaku apeti, 14/10/1997, Peña-Chocarro 361 (CTES); Paraguay, Cordillera, Valenzuela, 17/12/1950, Sparre and Vervoorst 1051, (LP); Paraguay, San Pedro, Primavera, 1957, Woolston 842 (SI).

Species not included

The spores of three species that inhabit the Southern Cone were not observed in this study for different reasons that are detailed below.

Ctenitis abyssi is a species known only for its type material and it is not available for palynological study. However, Viveros et al. (2018) who carried out the taxonomic monograph of the genus *Ctenitis* in South America, although they could access the material, they could not observe any spore.

Another is the case of *C. flexuosa*, a species that is known by few specimens with more than 100 years each, some from Rio de Janeiro and the other from Santa Catarina. The herbarium material is incomplete and characters such as rhizome and spores are unknown (Viveros et al. 2018).

Finally, the *C. laetevirens* specimens analyzed here showed aborted sporangia and it was not possible to observe any spores in them.

On the other hand, SEM in *C. fenestralis* was not performed because the material was very scarce.

RESULTS

Morphology

The measurements of the spores of the *Ctenitis* species analyzed are shown in Tables I and II.

The spores of the analyzed species of the genus *Ctenitis* in the Southern Cone of America are monolete, of bilateral symmetry and light to dark brown color. The outline is ellipsoidal or sub-circular in polar view and plane-convex to convex-subhemispheric in equatorial view.

C. ampla: The spores are light brown (Fig. 1a). Ellipsoidal to subcircular in polar view and plane-convex to convex-subhemispheric in equatorial view. The exospore is 0.9-1.2 µm thick and smooth. The laesura is delicate and due to the density of the sculptural elements it is not very visible (Fig. 2b). The sculpture is echinate (Figs. 2a, 2b), formed by conical spines, with a broad base and attenuated apex, of 3.9- 8.8 µm height, up to 1.2 µm wide at the apex and 3.1-5.7 µm wide base. The bases are coalescent with each other and this union can occur by small cords or not (Fig. 2c). The tips of the spines appear broken, showing that they are hollow inside (Fig. 2c).

C. anniesii: The spores are light brown (Fig. 1b). Ellipsoidal in polar view and plane to convex in equatorial view. The exospore is 0.5-0.7 µm thick and smooth. The sculpture is folded, formed by subglobose and sinuous folds of

Table I. ***Ctenitis*** **species spore measurements from Southern Cone of South America. Dimensions in µm.**

Species	MAED	MIED	PD	LL
C. ampla	35-49	28-42	28-38	8-19
C. anniesii	31-46	25-39	24-35	15-31
C. aspidioides	35-46	25-41	27-42	21-35
C. bigarellae	32-49	25-38	24-32	18-31
C. deflexa	29-39	18-28	18-28	11-28
C. distans	31-42	24-36	21-34	21-28
C. eriocaulis	34-42	24-32	27-34	14-17
C. falciculata	31-49	22-37	19-38	15-35
C. fenestralis	28-45	15-26	21-39	11-31
C. nervata	35-50	26-35	24-36	20-41
C. paranaensis	32-44	25-34	25-34	14-25
C. submarginalis	28-49	24-43	21-44	11-43

MAED: major equatorial diameter; MIED: minor equatorial diameter; PD: polar diameter; LL: laesura length.

variable dimensions (Fig. 2d, 2e). The perispore surface is rugulate-scabrate (Fig. 2f), formed by small rugulae and positive elements with a microgranulate appearance.

C. aspidioides: The spores are light brown (Fig. 1c). Ellipsoidal in polar view and plane to convex in equatorial view. The exospore is 0.5-0.7 μm thick and smooth. The sculpture is folded, formed by narrow, sparse folds, mostly long, which can be fused together (Fig. 2h). The perispore surface is scabrate between folds and finely scabrate on the folds (Fig. 2i). Some spheroids are observed with SEM on the perispore surface (Fig. 2g).

C. bigarellae: The spores are dark brown (Fig. 1d). Ellipsoidal in polar view and plane-convex to convex in equatorial view. The exospore is 0.6-0.7 μm thick and smooth. Folds associated to laesura are observed (Fig. 2k). The sculptures is folded, formed by short, subglobose to linear folds (Fig. 2j). The perispore surface is microverrucose (Fig. 2l).

C. deflexa: The spores are light brown (Fig. 1e). Ellipsoidal in polar view and plane to convex in equatorial view. The exospore is 0.3-0.5 μm thick and smooth. The sculpture is folded, formed by subglobose, linear and short folds (Figs. 3a, 3b). The perispore surface is scabrate between folds and psilated on folds (Fig. 3c).

C. distans: The spores are light brown (Fig. 1f). Ellipsoidal in polar view and plane-convex to convex in equatorial view. The exospore is 0.5-0.8 μm thick and smooth. The sculpture is folded, formed by linear and elongated folds and other short and subglobose ones (Figs. 3e). The perispore surface is scabrate between folds and psilate on the folds (Fig. 3f). Some spheroids are observed with SEM on the perispore surface (Figs. 3d).

C. eriocaulis: The spores are light brown (Fig. 1g). Ellipsoidal in polar view and plane-convex to convex in equatorial view. The exospore is 0.1-0.6 μm thick and smooth. Folds associated to the laesura are observed (Fig. 3h). The sculpture is folded, formed by short, subglobose and handle shape or coarse and elongated folds (Fig. 3g). The perispore surface is scabrate between folds and psilated on the folds apex (Fig. 3i).

C. falciculata: The spores are light brown (Fig. 1h). Ellipsoidal to slightly oblong in polar

Table II. Folds measurements of the rugated spores of *Ctenitis* from Southern Cone of South America. Dimensions in μm.

Species	Fold Length	Fold Width	Fold Height
C. anniesii	3-14.6	0.7-4.6	2.2-5.3
C. aspidioides	7-31.9	1.3-5.8	2.9-6.8
C. bigarellae	6.5-16.3	3-5	3.7-7
C. deflexa	4.7-10.1	1.6-4.9	3.3-5.7
C. distans	3.4-26.3	1.7-3.5	2.5-5.9
C. eriocaulis	3.5-9.7	1.5-6.3	3.4-7.4
C. falciculata	2.9-19.2	1.4-2.9	1.7-4
C. nervata	5.9-24.8	3.3-6.2	2.7-6.8
C. paranaensis	5.3-18.9	1-4	1.7-4.7
C. submarginalis	2.2-8.5	2.6-5.5	1.6-5

view and plane to convex in equatorial view. The exospore is 0.5-0.9 µm thick and smooth. Folds associated to the laesura are observed (Fig. 3k). The sculpture is folded, formed by elongated, subglobose, linear, sinuous and narrow folds (Fig. 3j). The perispore surface is coarsely scabrate between folds and finely scabrate on folds (Fig. 3l).

C. fenestralis: The spores are dark brown. Ellipsoidal in polar view and plane to convex in equatorial view. The sculpture is folded, formed by few low folds (Fig. 1i).

C. nervata: The spores are light brown (Fig. 1j). Ellipsoidal in polar view and plane to convex in equatorial view. The exospore is 0.6-0.7 µm thick and smooth. The sculpture is folded, formed by linear and narrow (Fig. 4a) or coarsely wide folds that are partially or totally fused (Fig. 4b). The perispore surface is coarsely scabrate between folds and finely scabrate on folds (Fig. 4c).

C. paranaensis: The spores are light brown (Fig. 1k). Ellipsoidal in polar view and plane-convex to convex in equatorial view. The exospore is 0.5-0.6 µm thick and smooth. Folds associated to the laesura are observed (Fig. 4e). The sculpture is folded, formed by linear and narrow, subglobose, or branched in Y-shape folds (Fig. 4d). The perispore surface is scabrate (Fig. 4f).

C. submarginalis: The spores are light brown (Fig. 1l). Ellipsoidal en polar view and plane to convex in equatorial view. The exospore is 0.3-0.9 µm thick and smooth. Folds associated to the laesura are observed (Fig. 4h). The sculpture is folded, formed by shorts, subglobose, rounded, handle-shape folds (Fig. 4g). The perispore surface is scabrate between folds and becomes finely scabrate/psilate on folds (Fig. 4i).

Ultrastructure

C. eriocaulis: the exospore is 0.19-0.61 µm thick, it is smooth and it is composed of two layers: the inner, 0.01-0.07 µm thick, is more contrasted that the outer one which is 0.4-0.54 µm thick (Fig. 5d). This wall widens at the base of the laesura (Fig. 5b). Simple and branched channels were observed in the outer exospore (Fig. 5c).

The perispore is 0.19-0.86 µm thick and it is composed of two layers. The inner layer is 0.07-0.6 µm thick, more contrasted than the exospore and forms the ornamentation of the spore (Fig. 5a). The outer layer is 0.02-0.27 µm thick, it is less contrasted than the inner one and it covers the folds on their outside as well as inside (Fig. 5f). Several small portions of membranes (scales) are in the perispore inner layer (Fig. 5e). The laesura usually has an associated supralaesural fold (Fig. 5b).

C. submarginalis: the exospore is 0.36-0.93 µm thick, it is smooth and it is composed of two layers (Fig. 6d): the inner, 0.02-0.07 µm thick, is more contrasted that the outer one which is 0.29-0.92 µm thick. This wall widens at the base of the laesura (Fig. 6b). Simple and branched channels were observed in the exospore (Fig. 6e).

The perispore is 0.25-1.24 µm thick and it is composed of two layers (Fig. 6c). The inner layer is 0.22-1.19 µm thick, more contrasted than the exospore and forms the ornamentation of the spore (Fig. 6a). The outer layer is 0.01-0.12 µm thick, it is less contrasted than the inner one and it covers the folds on their outside as well as inside. Several small portions of membranes (scales) are in the perispore inner layer (Fig. 6f).

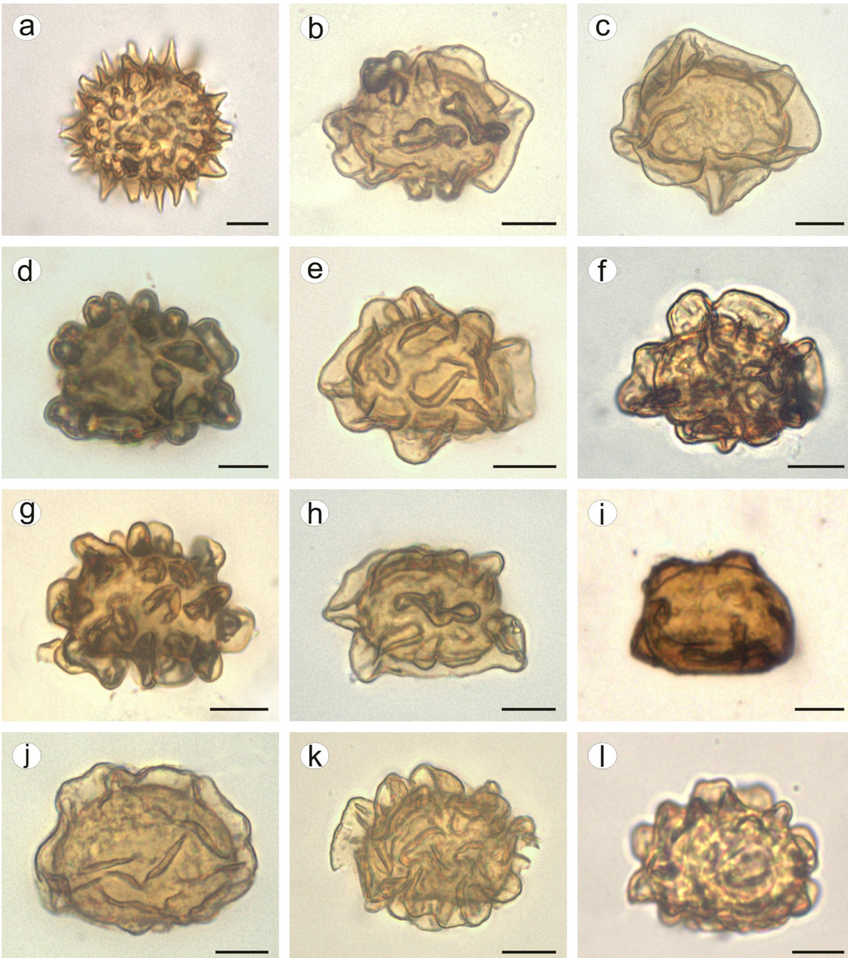

Figure 1. Spores of *Ctenitis* with LM. **a.** *C. ampla*. **b.** *C. anniesii*. **c.** *C. aspidioides*. **d.** *C. bigarellae*. **e.** *C. deflexa*. **f.** *C. distans*. **g.** *C. eriocaulis*. **h.** *C. falciculata*. **i.** *C. fenestralis*. **j.** *C. nervata*. **k.** *C. paranaensis*. **l.** *C. submarginalis*. Scale bars: a-l = 10 µm.

DISCUSSION

The characteristics of the spores seen in LM, such as color, shape and ornamentation, provide relevant information for various disciplines, such as palaeobotany, palaeoecology, aerobiology and forensic palynology (Farfán-Santillán et al. 2016). Likewise, Gorrer et al. (2021) differentiated ferns genera and even some species using the characteristics of the spores under LM.

The only analysis with LM for folded species prior to the one carried out in this work was carried out by Lebrão et al. (2014) with three *Ctenitis* species that inhabit the Southern Cone: *C. aspidioides*, *C. distans* and *C. falciculata*. Illustrations in this study show that *C. falciculata* has mainly compressed or inflated folds, others short and handle-shaped that differ considerably from those found here, resembling that observed for the spores of *C. bigarellae* and *C. submarginalis* analyzed here.

Murillo & Bless (1978) described and illustrated the spores of *C. ampla* from Colombia with LM. These authors indicate that the spines are up to 8 µm high, a value similar to that found in this work (8.8 µm), although they do not describe the spines characteristics.

The color of the spores of the genus is a characteristic not mentioned by any of the authors in previous works. Only Lebrão et al. (2014) illustrate with LM the acetolyzed spores of 3 species of *Ctenitis* from central Brazil, where it is observed that they are light brown in color. In any case, most of the species that inhabit the

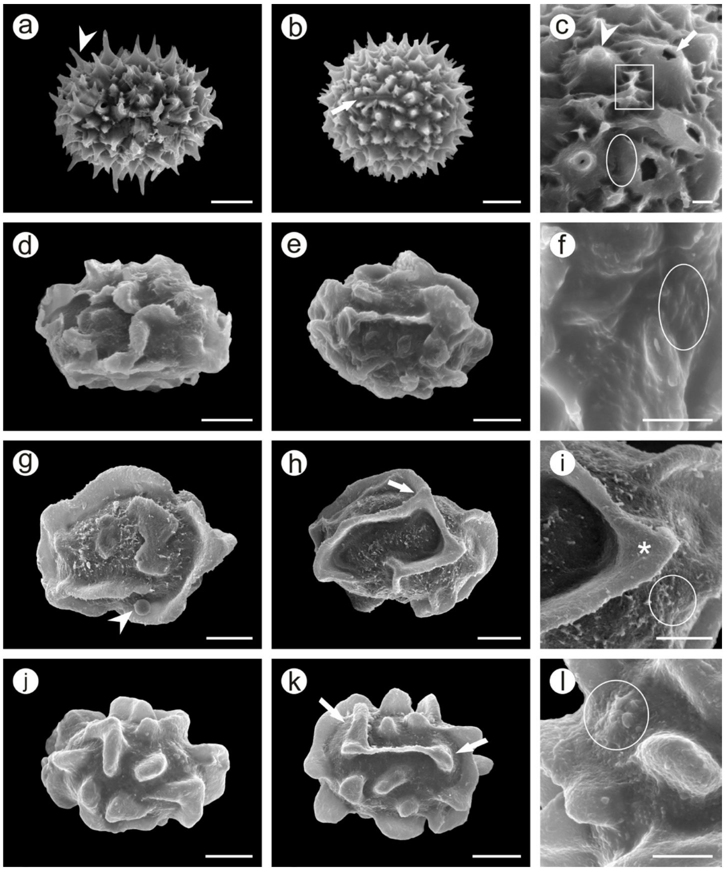

Figure 2. Spores of *C. ampla*, *C. anniesii*, *C. aspidioides* and *C. bigarellae* with SEM. **a-c**: *C. ampla*. **a.** Spore in equatorial view with conical spines with broad bases and attenuate apex (arrowhead). **b.** Spore in proximal view with coarse spines. The arrow shows the delicate laesura. **c.** Perispore surface. The arrowhead shows a spine apex broken. The spine is hollow inside (arrow). Spine bases are connected by cords (square) or without it (circle). **d-f**: *C. anniesii*. **d.** Spore in equatorial view with subglobose and sinuous folds. **e.** Spore in proximal view with short and subglobose folds. **f.** Rugulate-scabrate surface of the perispore (circle). **g-i**: *C. aspidioides*. **g.** Spore in equatorial view with long folds in the edges. A spheroid is observed at the perispore surface (arrowhead). **h.** Spore in distal view with narrow and elongated folds. Some folds are fused (arrow). **i.** Scabrate perispore surface between folds (circle) and finely scabrate on the folds (asterisk). **j-l**: *C. bigarellae*. **j.** Spore in equatorial view with short, inflated and subglobose folds. **k.** Spore in polar view with short folds associated to the laesura (arrows). **l.** Microverrucose perispore surface (circle). Scale bars: **a, b, d, e, g, h, j, k** = 10 μm; **c, f** = 2 μm; **i, l** = 5 μm.

Southern Cone are brown or light brown except for *C. bigarellae*, which is dark brown.

Regarding SEM studies, Tryon & Lugardon (1991) illustrated only two species with folded spores, *C. velata* R. M. Tryon & A. F. Tryon from Cuba and *C. submarginalis* from Brazil with inflated, short and subglobose folds. These observations are similar to those found in the specimens analyzed here for the Southern Cone of America.

The spores of *C. ampla* illustrated here with SEM with material from Argentina, show a perispore formed by coarse spines, similar to that illustrated by Moy (1988) with Venezuelan material. In addition, the illustrations by Tryon & Lugardon (1991) of *C. sloanei* (Poepp. ex Spreng.) C. V. Morton from El Salvador, *C. catocarpa* (Kunze.) C. V. Morton from Peru (synonyms of *C. ampla*), *C. hirta* from Jamaica and *C. nigrovenia* (Christ) Copel. from Colombia show an echinated sculpture. In the illustrations of these authors, as in those of this work, it can be seen that the spines are coarse and have a broad base and usually broken apex, revealing that they are hollow inside. The widened base of a spine without its apex reveals a hole, which is called a “tubercle” by Tryon & Lugardon (1991). This could be due to its appearance of a perforated tubercle, but the term “tubercle” can be confused since they can be massive, therefore, it is suggested to use the term “fold” to avoid confusions of this nature.

In a floristic work of the genus *Ctenitis* for South America, Viveros & Salino (2015) show illustrations with SEM of the spores of *C. christensenii* R. S. Viveros & Salino. The spores of this species have compressed and

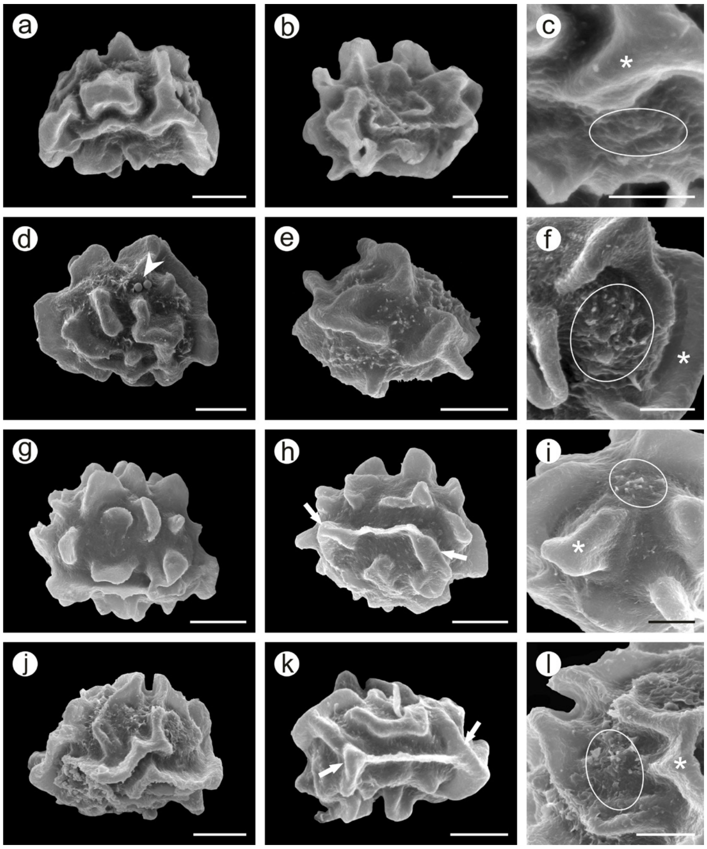

Figure 3. Spores of *C. deflexa*, *C. distans*, C. *eriocaulis* and *C. falciculata* with SEM. a-c: *C. deflexa*. a. Spore in equatorial view with short and subglobose folds. b. Spore in proximal view with short and inflated folds. c. Scabrate perispore surface between folds (circle) and psilated on the folds (asterisk). d-f: *C. distans*. d. Spore in equatorial view with elongated and short folds. Spheroids are observed at the perispore surface (arrowhead). e. Spore in proximal view with linear and elongated folds. f. Scabrate perispore surface between folds (circle) and psilated on the folds (asterisk). g-i: *C. eriocaulis*. g. Spore in equatorial view with short and subglobose folds. h. Spore in proximal view with narrow and inflated folds and other associated to the laesura (arrows). i. Scabrate perispore surface between folds (circle) and psilate on the folds (asterisk). j-l: *C. falciculata*. j. Spore in equatorial view with linear and elongated folds. k. Spore in polar view with sinuous and subglobose folds and other associated to the laesura (arrows). l. Coarsely scabrate perispore surface between folds (circle) and finely scabrate on the folds (asterisk). Scale bars: a, b, d, e, g, h, j, k = 10 μm; c, f, l = 5 μm; i = 3 μm.

handle-shaped folds, similar to those found in this study for *C. submarginalis*.

Viveros et al. (2018) affirm that the rugated spores have coarse folds and also present tubercles that can vary from small to large. However, according to some species analyzed here, such as *C. bigarellae* and *C. submarginalis*, we considered that they have a perispore with rather short, inflated, subglobose, or handle-shaped folds. Other cases are *C. aspidioides* and *C. nervata*, which, unlike the analysis by Viveros et al. (2018), the spores observed here present very elongated folds.

With respect to the perispore surface, Tryon & Lugardon (1991) argue that folded spores of the genus *Ctenitis* rarely have a finely reticulated surface. However, no species of the genus within the Southern Cone of America observed here presents a finely reticulated surface. The remaining South American species analyzed by Viveros et al. (2018) do not present this pattern either.

The spine bases of *C. hirta* illustrated by Tryon & Lugardon (1991) with material from Jamaica, are connected by cords. In the spores observed here of *C. ampla*, with material from Argentina, these cords may or may not be present. Thus, on the surface of echinated spores, the coalescence or not of the spine bases seems to be a variable characteristic in American species.

In a floristic contribution, Ponce & Martínez (2012) mentioned that the spores of the genus *Ctenitis* in northwestern Argentina, have an echinulated surface. In this region of the country *C. ampla* and *C. submarginalis* coexist, and neither of the two species present this type of perispore surface, since the first presents coarse echinae and the second presents a folded perispore, formed by folds and a scabrated surface.

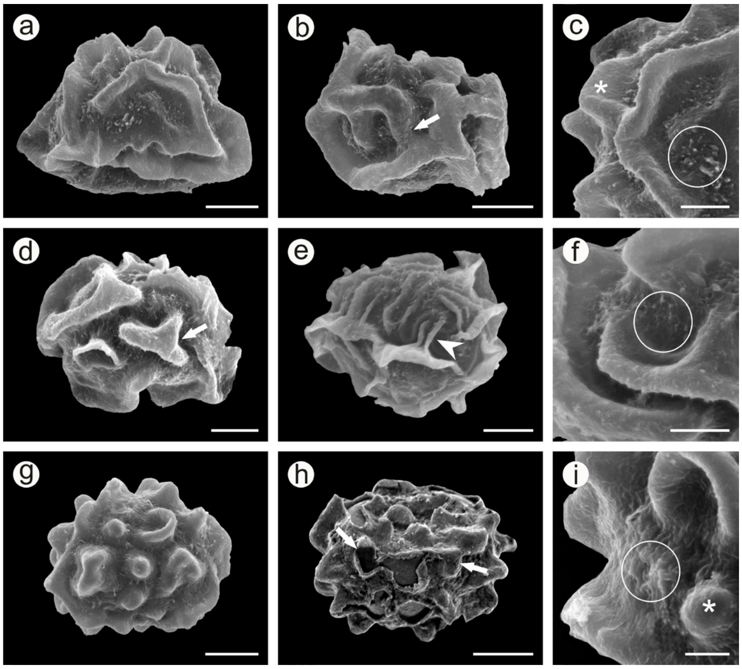

Figure 4. Spores of *C. nervata*, *C. paranaensis* and *C. submarginalis* with SEM. **a-c**: *C. nervata*. **a.** Spore in equatorial view with narrow and elongated folds. **b.** Spore in distal view coarse folds partially fused (arrow). **c.** Coarsely scabrate perispore surface between folds (circle) and finely scabrate on folds. **d-f**: *C. paranaensis*. **d.** Spore in equatorial view with subglobose and narrow folds. The arrow shows Y-shape fold. **e.** Spore in proximal view with linear and elongated folds and other associated to the laesura (arrowhead). **f.** Scabrate perispore surface (circle). **g-i**: *C. submarginalis*. **g.** Spore in equatorial view with short, handle-shape and subglobose folds. **h.** Spore in polar view with short and inflated folds and folds associated to the laesura (arrows). **i.** Scabrate perispore surface between folds (circle) and psilate on the folds (asterisk). Scale bars: **a, b, d, e, g, h** = 10 μm; **c, f** = 5 μm; **i** = 3 μm.

The groups "*C. submarginalis*", "*C. ampla*" and "*C. hirta*" were proposed for the Neotropics by Christensen (1913, 1920) and Stolze (1990) focused in morphological characteristics. Later, this groups were phylogenetically tested with a limited sampling by Hennequin et al. (2017) and showed that were well supported. In relation to these groups, Viveros et al. (2018) pointed out that the South American species shows congruence between ornamentation patterns of spores and the degree of division of the lamina practically in their entirety. South American species with echinate spores are also those that have lamina with the highest degree of division (2-4-pinnate-pinnatifid = "*C. ampla*" group), including *C. ampla* and excepting *C. nigrovenia*, which has a 1-pinnate-pinnatifid lamina ("*C. submarginalis*" group). While the species with folded spores are those that are found in species with leaf of a lesser degree of division (1-pinnate-pinnatifid = "*C. submarginalis*" group). Regarding the species that inhabit the Southern Cone, the relationship between the morphology of the spores and the degree of division of the lamina has been confirmed in full this study. In this way, the sculpture of the spores can be considered as a diagnostic character within the genus *Ctenitis*, evidencing natural groups of species.

Although Walker (1966, 1973) and Smith & Mickel (1977) have corroborated that hybrids are not recorded in *Ctenitis* from Jamaica and Mexico, respectively, the sporangia of some South American species analyzed by Viveros et al. (2018) were found stunted and aborted. Such is the case of *C. abyssi*, *C. aspidioides*, *C. fenestralis* and *C. laetevirens* and due to this, specimens of these species presented deformed spores, poorly developed or were not found at all. For the species analyzed in this work, all the analyzed specimens of *C. aspidioides* turned out to be normal, with sporangia and developed spores. While, with *C. fenestralis* Viveros et al. (2018) reported that the spores lacked maturity, were deformed or aborted, being solitary or forming clusters. The same spore characteristics was observed by us in the studied material. Likewise, the specimens of *C. laetevirens* analyzed here were completely devoid of

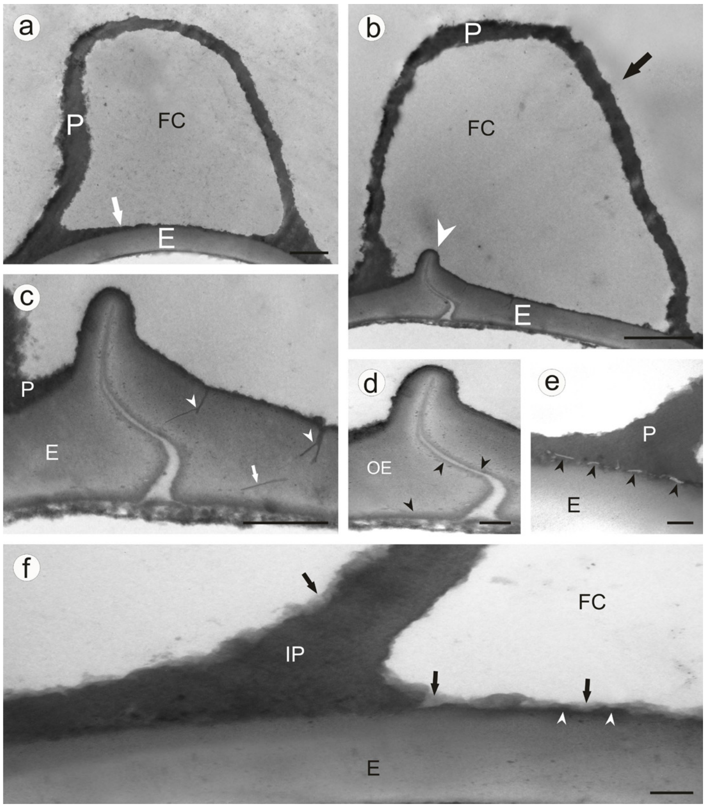

Figure 5. Spores of *C. eriocaulis* with TEM. a. Section through the sporoderm. The smooth exospore (E) is observed and the perispore (P) forms the ornamentation. The arrow shows the base of the fold. FC: fold cavity. b. Section through the supralaesural fold (arrow). The exospore (E) widens where the laesura emerges (arrowhead). P: perispore; FC: fold cavity. c. Section through the laesura. Simple (arrow) and branched (arrowhead) channels are seen in the exospore (E). P: perispore. d. Section through the sporoderm. The inner exospore (arrowheads) is very thin and more contrasted than the outer exospore (OE). e. Numerous scales (arrowheads) are seen at the base of the perispore (P) near the exospore (E). f. The smooth exospore (E) is observed. The perispore is made up of 2 layers: the inner perispore (IP) that forms the ornamentation and the outer perispore (arrows) that covers the internal and external surfaces of the fold. The arrowheads indicates the fold basis. FC: fold cavity. Scale bars: a = 1 µm; b, f = 0.5 µm; c, e = 0.2 µm; d = 0.1 µm.

spores in their stunted and blackish sporangia. These characteristics of atrophied and blackish sporangia or immature, deformed or aborted spores are typical of hybrid taxa or with problems of sporogenesis, a topic in which the genus has not been studied in depth. However, in sister genera such as *Dryopteris* Adans. (Montgomery 1982) and *Arachniodes* Blume (Widén et al. 1981), hybrids are recurrent.

Regarding TEM studies, in the folded spores of *C. eriocaulis* y *C. submarginalis* analyzed here, it is evidenced that the perispore is formed by folds that are hollow inside, just like the spores of *C. strigilosa* (Davenp.) Copel. illustrated by Tryon & Lugardon (1991) with Mexican material. On the other hand, Moy (1988) illustrates with TEM the spores of *C. ampla* with material from Venezuela, suggesting that the spines are perispore structures and are massive. This author affirms that the perispore in this ornamental type is formed by a single layer. It should be noted that the illustrations analyzed here with SEM and those made by Tryon & Lugardon (1991) clearly show that the spines of this species are hollow and not solid.

The ultrastructural characteristics of the spores analyzed by Gorrer et al. (2020) for the genus *Dryopteris* species that inhabit Argentina are similar to those analyzed here.

In this study, some channels were observed mainly in the outer exospore and they may end in the inner exospore. However, neither Moy (1988) nor Tryon & Lugardon (1991) have observed these structures in their respective studies.

Spheroids were observed with SEM in *C. aspidioides* and *C. distans*, however, since they could not be observed with TEM, it cannot be determined whether they are globules or spherules, as indicated by Tryon & Lugardon (1991).

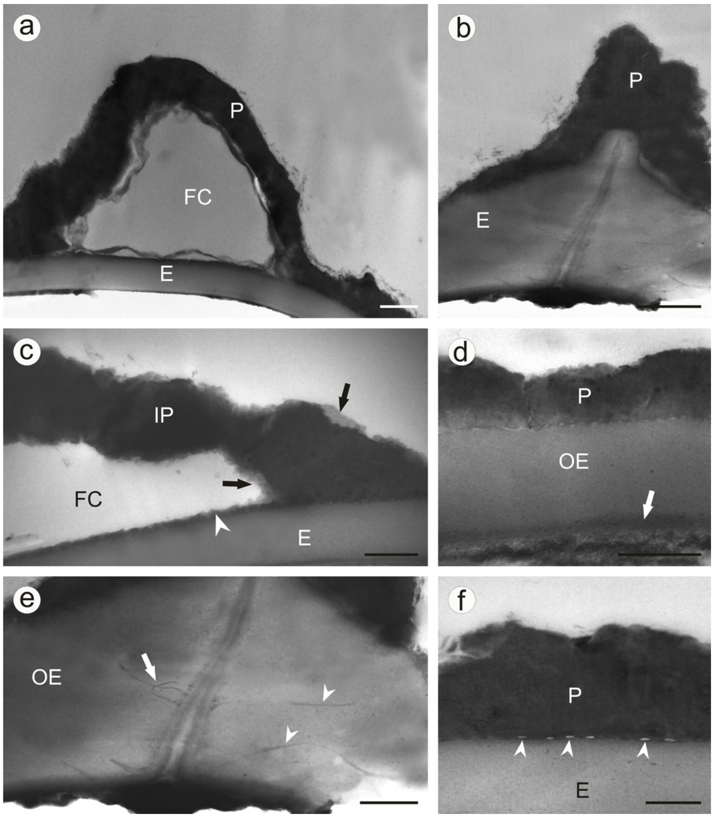

Figure 6. Spores of *C. submarginalis* with TEM. a. Section through the sporoderm. The smooth exospore (E) is observed and the perispore (P) forms the ornamentation. FC: fold cavity. b. Section through the laesura. The exospore (E) widens where the laesura emerges (arrowhead). P: perispore. c. Section through the sporoderm. The smooth exospore (E) is observed. The perispore is made up of 2 layers: the inner perispore (IP) that forms the ornamentation and the outer perispore (arrows) that covers the internal and external surfaces of the fold. The arrowheads indicates the fold basis. FC: fold cavity. d. The inner exospore (arrow) is very thin and more contrasted than the outer exospore (OE). P: perispore. e. Section through the laesura. Simple (arrowheads) and branched (arrow) channels are seen in the exospore. OE: outer exospore; L: laesura. f. Numerous scales (arrowheads) are seen at the base of the perispore (P) near the exospore (E). Scale bars: a, b = 1 µm; c, d = 0.3 µm; e, f = 0.5 µm.

CONCLUSIONS

The sculpture of the spores in the *Ctenitis* species are echinate or folded. Species with folded spores are those that also have a 1-pinnate-pinnatifid lamina, such as "*C. submarginalis*" group, while *C. ampla* is the only species within the genus in the Southern Cone of America representative of the group with echinated spores and a 2-4-pinnate-pinnatifid lamina.

The species that share the folded type have very similar macro and micro-ornamentation. The macro-ornamentation is made up of folds with variable dimensions. Regarding their morphology, they can be linear, sinuous, subglobose, handle-shape, which can be regularly or not distributed throughout the spore. The folds may be partially fused and in some cases they may have Y-shaped branches. Folds were found associated with the laesura. Macro-ornamentation is the characteristic with the greatest variability, both quantitatively and qualitatively, within the folded type. The perispore surface is similar in most species. It is microverrucous in *C. bigarellae*, rugulate in *C. anniesii*, or light to densely scabrate between the folds, and psilate to finely scabrate on the folds.

The echinate type was observed only in the spores of *C. ampla*. The ornamentation is made up of conical spines, with a wide base and an attenuated apex, hollow inside. The base of the spines can be smooth or have cords that connect with the bases of other spines.

The color of the spores observed at LM varies from light to brown, with the exception of *C. bigarellae*, in which case the color of its spores is dark brown.

Stratification and ultrastructure in the species analyzed in the genus are very similar. The sporoderm is made up of a smooth exospore and a perispore which forms the ornamentation of the spores constituted by hollow folds. The exospore and the perispore are made up of two layers that can be distinguished by thickness and contrast. In the exospore, the inner layer is thinner and more contrasted than the outer. In the perispore, the inner layer is the widest and is the one that forms the ornamentation while the outer layer is thinner, less contrasted and is the one that covers the internal and external surfaces of the folds. In *C. eriocaulis*, it was observed that the laesura is covered by a supralesural fold, a characteristic not observed in *C. submarginalis*. Simple and branched channels were observed, mainly in the outer exospore. Numerous scales were observed immersed in the base of the perispore where this wall borders the exospore.

Through palynological analysis it was possible to find spores in immature sporangia of *C. fenestralis*, in solitary forms or forming tetrad-like aggregations lacking complete formation, presenting a few folds in some spores. On the other hand, the analyzed *C. laetevirens* sporangia in all samples were found to be completely aborted, blackish and without spores.

The characteristics of these spores provide relevant information to differentiate some species or groups of species within the genus in the Southern Cone of America, such as echinated spores in *C. ampla*, dark brown spores in *C. bigarellae* and spores with long folds in *C. aspidioides* and *C. nervata*. In this way, the immature spores in *C. fenestralis* and the aborted sporangia in *C. laetevirens* could help to detect possible problems in sporogenesis.

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Daniel Alejandro Gorrer: Collection, Analysis, figure preparation and Writing the manuscript; Juan Pablo Ramos Giacosa and Gabriela Elena Giudice: Analysis, Writing of manuscript and Manuscript revision.