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Ferns and Lycophytes as new challenges

Palynomorphometry in ferns: *Ctenitis* (Dryopteridaceae), a case of study in the Southern Cone of America

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Abstract

The morphometric knowledge about the spores of the *Ctenitis* has not yet been deepened. Considering the folded spores of this genus which inhabit the Southern Cone of America the aims were to increase infrageneric knowledge, to evaluate the relationship among the morphometric characters of the folded spores in these *Ctenitis* species and to verify the spore's morphometry as a taxonomic tool. This study was performed with herbarium material. The spores were analyzed with light microscopy (LM) and scanning electron microscopy (SEM). The variables analyzed were: major and minor equatorial diameter, polar diameter, length of the laesura and length, width and height of the folds. The test chosen to analyze the study variables was ANOVA. Four spore sets are proposed. The "elongate-type" is made up of *C. aspidioides* and *C. nervata*. The "medium-type" is made up of *C. anniesii*, *C. distans*, *C. falciculata* and *C. paranaensis*. The "handle shape-type" is represented by *C. bigarellae*, C. *eriocaulis* and *C. submarginalis.* Finally, the "short-type", consists only of *C. deflexa*. The morphometric data of spores together with the statistical analysis provided useful information for the distinction of some *Ctenitis* species studied in the Southern Cone of America. These results contribute to the studies aeropalynologycal and palaeopalynological.

Key words: *Ctenitis*, ferns, morphometry, palinology, spore morphology.

Resumen

El conocimiento morfométrico sobre las esporas de la *Ctenitis* aún no se ha profundizado. Considerando las esporas plegadas de este género que habitan el Cono Sur de América, los objetivos de este estudio fueron aumentar el conocimiento infragenérico, evaluar la relación entre los caracteres morfométricos de las esporas plegadas en estas especies de *Ctenitis* y verificar la morfometría de las esporas como herramienta taxonómica. Este estudio se realizó con material de herbario. Las esporas se analizaron con microscopia óptica (MO) y microscopía electrónica de barrido (MEB). Las variables analizadas fueron: diámetro ecuatorial mayor y menor, diámetro polar, largo de lesura y largo, ancho y alto de los pliegues. La prueba elegida para analizar las variables de estudio fue ANOVA. Se proponen cuatro conjuntos de esporas. El "tipo-alargado" está formado por *C. aspidioides* y *C. nervata*. El "tipo-medio" está compuesto por *C. anniesii*, *C. distans*, *C. falciculata* y C. *paranaensis*. El "tipo-forma de mango" está representado por *C. bigarellae*, *C. eriocaulis* y *C. submarginalis*. Finalmente, el "tipo-corto", consiste solo en *C. deflexa*. Los datos morfométricos junto con el análisis estadístico brindaron información útil para la distinción de algunas especies de *Ctenitis* estudiadas en el Cono Sur de América. Estos resultados contribuyen a estudios aeropalinológicos y paleopalinológicos.

Palabras clave: *Ctenitis*, helechos, morfometría, palinología, morfología de esporas.

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Introduction

After seed-bearing plants, ferns and lycophytes are the most diverse group of vascular plants, containing around 12,000 (PPGI 2016) species which are distributed worldwide. According to Tryon & Lugardon (1991), ferns and lycophytes spores are single airborne cells with complex walls. Palynological records of these groups provide not only stratigraphic data and evidence of vascular plants' early diversification, but also testimony of climatic and vegetational changes. Two basic forms are recognized in terms of shape: the monolete and the trilete. The first is usually bilaterally symmetrical and ellipsoidal with a linear aperture. The second is mostly radially symmetrical, tetrahedral; and it possesses a globose hemispheric distal face with a proximal triradiate aperture (Tryon & Lugardon 1991). Spore morphological features have been pivotal; they played a vital role in the assessment, classification, identification and phylogenetic relationships of ferns from all taxonomic ranks (Adeonipekun *et al.* 2021).

Spore morphology of fern species has been observed and described under light microscopy (LM) and scanning electron microscopy (SEM) in previous studies (*e.g.*, Nayar & Devi 1964; Gastony 1974; Morbelli 1976; Tryon & Lugardon 1991; Giudice *et al.* 2004; Moran *et al.* 2007; Ramos Giacosa *et al.* 2015, 2016; Gorrer *et al.* 2020, 2022). In these studies, differences in shape, size, aperture characters and wall ornamentation were reported. The importance of spore morphological studies is evidenced by their use in resolving disparities in the classification of ferns (Triana-Moreno 2012; Yáñez *et al.* 2016). The application of this micromorphological data also extends to other fields such as allergology (Simán *et al.* 1999), archaeology (Kvavadze *et al.* 2019), climatology (Barboni *et al.* 2004), forensic palynology (Povilauskas 2019) and fern sporulation phenology (Adeonipekun *et al.* 2019). Spores are complementary tools used to generate useful and reliable micro-morphometric data in taxonomic studies of fern families (Moran *et al.* 2007; Yáñez *et al.* 2016, 2017; Shah *et al.* 2018; Triana-Moreno 2022). This data has enhanced the understanding of the shape, size, and sculpturing of lycophytes and ferns spore walls as well as aided in the identification of interspecific differences among spores of different ferns at any scale (Adeonipekun *et al.* 2021).

In ferns, spore size and morphology have long been useful for systematic and evolutionary studies (Tryon & Lugardon 1991; Giudice *et al.* 2004; Yañez *et al.* 2017; Ramos Giacosa 2019). Furthermore, spore morphology is useful to determine relationships among taxa higher than the species level, such as in genus delimitation (Chao & Huang 2018; Morajkar *et al.* 2021). Spore surface ornamentation has also shown to be informative and useful for phylogenetic studies (Chen *et al.* 2021). Barrington *et al.* (1986) laid the foundations for the modern of quantitative methods used in analyzing spore size differences among closely related species. Recent work has also included statistical analysis of spore size data as a standard component of the strategy used to infer the evolutionary histories of lineages, including polyploids in ferns (Sigel *et al.* 2011). Much of the potential utility in measuring spore size remains unexplored. Furthermore, there is virtually no quantitative data on the shape of fern spores in the existing literature except for the diameters (Nayar & Devi 1964; Marquez 2010; Silva *et al.* 2019; Pérez-Jiménez *et al.* 2020). Spore size, as it has been represented until now, conflates size and shape because of the common practice of providing the measurement of only one dimension per spore. In the case of monolete spores, the "size" measure is actually the length. According to Barrington *et al.* (2020) spore size is defined as the space occupied by the spore. In this sense, the spore projections are practically an unexplored area (Barrington *et al.* 2020).

Multivariate analyses of ferns and lycophytes have been carried out to explore and determine relevant characters in order to separate species groups and support the recognition of species (Wei & Zhang 2013; Shah *et al.* 2019; Magrini & Scoppola 2010; Petchsri *et al.* 2012). Some of the characters included in multivariate analyses for segregating ferns and lycophytes species are: blade, stipe, and petiolule features; the shape of rachis scales, the indusial margin, the number of sporangium cells; and the color, shape and diameter of spores (Hernández-Hernández *et al.* 2009). Multivariate analyses can be used to recognize groups of species or populations. They can also be useful in detecting characters which allow for the separation of species or groups of species as shown by Hernández-Hernández *et al.* (2009) who, with the aid of multivariate analyses, could distinguish between *Dryopteris rossii* C. Chr. and *D. wallichiana* (Spreng.) Hyl., species belonging to the *D. patula* (Sw.) Underw. complex.

A few studies, carried out by Coelho & Esteves (2011), Ramírez-Valencia *et al.* (2013), Duarte *et al.* (2014) and Ramírez-Valencia & Sanín (2017) have demonstrated that statistical analysis of the measurements and other parameters showed significant variation and could be used to obtain a correct description and identification of several types of Anemiacean and Polypodiacean spores.

According to PPG I (2016), the family Dryopteridaceae has almost 2,100 species. *Ctenitis* (C. Chr.) C. Chr. is one of the most diverse genera within the family since it has around 125 species which are distributed throughout the humid tropics in the world (Viveros & Salino 2015). It is characterized by the presence of catenate trichomes (ctenitoid hairs) on the adaxial surfaces of its petioles, rachises and coastae; and by the attenuated and thin termination of the veins (Viveros & Salino 2015, 2017).

For the Southern Cone of America, 15 species of the genus *Ctenitis* are cited (Zuloaga *et al.* 2019; Viveros & Salino 2023). However, two of them present aborted spores, and another two show spores not seen so far (Viveros *et al.* 2018). Of the remaining 11, only one has equinated spores and the other ten species have folded spores (Gorrer *et al.* 2022).

Since morphometric knowledge about the spores of the genus has not yet been deepened, the proposal of this work is the incorporation of new micromorphological characters to provide more evidence for the delimitation and/or grouping of species in the genus. Considering the morphology of the folded spores of the *Ctenitis* which inhabit the Southern Cone of America and complementing the study carried out by Gorrer *et al.* (2022), the aims of this study are: 1) to increase infrageneric knowledge, 2) to evaluate the relationship among the morphometric characters of the folded spores in these *Ctenitis* species, and 3) to verify the spores morphometry as a taxonomic tool.

Matherial and Methods

For this study, herbarium materials from the following national and international institutions were used: BA, BCRU, BHCB, CTES, LP, RB and SI (Thiers, continuously updated). The specimens consulted are detailed below.

Ctenitis anniesii (Rosesnst.) Copel.: BRASIL. RIO DE JANEIRO: Itatiaia, Parque Nacional de Itatiaia, Maromba, 21.III.1942, *Barros 707* (RB). PARANÁ: Tunas do Paraná, colonia Joao XXIII, 2002, *Ribas y Abe 4718* (SI).

Ctenitis aspidioides (F.M. Bailey) Copel.: BRASIL. RIO DE JANEIRO: Santa Maria Madalena, Serra da Grama, 24.XI.1977, *Mautone 450* (RB).

Ctenitis bigarellae Schwartsb., Labiak & Salino: BRASIL. MINAS GERAIS: Catas Altas, RPPN Santuário do Caraça, caminho para o Pico da Conceição, 26.VIII.2008, *Viveros*, *Salino*, *Oliveira y Giacomim 22* (BHCB).

Ctenitis deflexa (Kaulf.) Copel.: BRASIL. RIO DE JANEIRO: Nova Friburgo, California, 19.XI.1922, *Kuhlmann 110* (RB).

Ctenitis distans (Brack.) Ching: BRASIL. SÃO PAULO: Iporanga, Apiaí, Parque Estadual Turístico do Alto Ribeira (PETAR), Núcleo Caboclos, 22.VIII.2012, *Mazziero y Engels 1168* (RB).

Ctenitis eriocaulis Alston: BRASIL. ALAGOAS: São José da Lage, Usina Serra Grande, Mata Maria Maior, Grota do Gereba, 2001, *Pietrobom 5333* (BHCB).

Ctenitis falciculata (Radii) Ching: BRASIL. SANTA CATARINA: Armação do Sul, 15.XII.1947, *Senhem 3156* (RB).

Ctenitis nervata (Fée) R.S. Viveros & Salino: BRASIL. PARANÁ: Morretes, Estaçao Marumbi, 2.I.1986, *Kummrow y Cordeiro 2701* (CTES). SANTA CATARINA: Biguaçu, Fechinal, 18.I.1945, *Reitz C1004* (RB). Joinville, Entrada Dona Francisca, 1906, *Rosenstock 118a* (SI).

Ctenitis paranaensis (C. Chr.) Lellinger: BRASIL. SANTA CATARINA: Meleiro-Graranguá, 13.X.1943, *Reitz 11* (RB).

Ctenitis submarginalis (Langsd. & Fisch.) Ching: ARGENTINA. TUCUMÁN: Villa Nogués, IX.1911, *Lillo 12007* (BA). MISIONES: Guaraní, Predio Guaraní, tramo II, 25.XI.1993, *Tressens*, *Cristobal*, *Khel et al. 4741* (LP). BUENOS AIRES: Ensenada, Reserva Natural Punta Lara, 2006, *Ramos Giacosa 24* (LP). BRASIL. RIO GRANDE DO SUL: Canguçú, 20.VI.1968, *Ceroni 4948* (CTES). PARANÁ: município de Curitiba, Capanema, 13.XI.1973, *Kummrow 98* (LP). PARAGUAY. CANINDEYÚ: Jeju-mi, sendero Jaku apeti, 14.X.1997, *Peña-Chocarro 361* (CTES).

Morphometry can be defined as the analysis of any biological form (Henderson 2006). To refer to the analysis of measurements and other quantitative parameters in spores, I propose to use the term "palynomorphometry".

The Southern Cone of America, according to Zuloaga *et al.* (2019) includes all the territories of Argentina, Chile, Paraguay, Uruguay and the three southern states of Brazil (Rio Grande do Sul, Santa Catarina and Paraná).

The spores were analyzed with LM and SEM. For LM analysis, acetolysis was performed (Erdtman 1960). For SEM, the spores without treatment were placed into stubs with adhesive double-faced tape and coated with gold. Measurements were taken with the ImageJ program, by means of photographs taken with LM and SEM. The LM observations were made with a Nikon E200 from Cátedra de Morfología Vegetal, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata. The photographs of SEM material were taken with a JEOL JSMT-100 SEM from Museo de Ciencias Naturales de La Plata. The measures of acetolyzed spores were randomly estimated on 20 spores in each sample.

Statistical analysis was performed with the Statgraphics Centurion XVI program (Guerrero Martín *et al.* 2014; Milla *et al.* 2020). The variables analyzed were the following seven continuous quantitative characters: major equatorial diameter (MAED), minor equatorial diameter (MIED), polar diameter (PD), length of the laesura (LL), length of the fold (LF), width of the fold (WF) and height of the fold (HF). The test chosen to analyze the study variables was the analysis of variance (ANOVA) (St & Wold 1989). The objective of running this test was to establish relevant differences among the species to be studied by comparing the means of each variable analyzed. The analysis methodology consisted of first, evaluating the following two assumptions: on the one hand, normality of the residuals and on the other, homoscedasticity of the variances. These assumptions had to be validated by the Shapiro-Wilk (Hanusz *et al.* 2016) and Levene tests (Gastwirth *et al.* 2009), respectively. The hypothesis started in each case is that residuals must have a normal distribution and variances within each group do not differ from each other. The hypothesis test is detailed below.

In both cases, the null hypothesis had to be accepted to validate the assumptions. Thus the probability had to exceed the alpha value (0.05). When the assumptions failed to be validated, a transformation was applied to the data using the natural logarithm (Ln) so that the variable in question could reduce the intragroup variability. Later, the validation of the assumptions with the transformed variable was retested. Once the assumptions had been validated, the analysis of variance was carried out; in this case, the hypothesis to be tested was that the means of all the groups analyzed were equal to each other.

When the probability value was lower than the alpha (<0.05), the null hypothesis was rejected thus corroborating that there were statistically significant differences among some means. Afterwards, a post hoc test was carried out in order to elucidate which were the means which differed from each other. The test chosen to be run was the LSD test (Williams & Abdi 2010). In the event that the assumptions of the normality of the residuals and the homoscedasticity of the variances had not been validated, even with the variable transformed by the natural logarithm (Ln), a non-parametric test was carried out to conclude the analysis. In this case, the test chosen was Kruskal-Wallis (Ostertagová *et al.* 2014) in which the hypothesis to be tested was that the medians of the groups analyzed did not differ from each other.

When the probability value was lower than alpha (< 0.05), the null hypothesis was rejected, corroborating that there were statistically significant differences between some medians. Subsequently, a post hoc test was conducted in order to elucidate which were those medians that differed from each other. The test chosen was the Bonferroni test (Bland & Altman 1995).

Afterwards, a principal component analysis (PCA) (Ringnér 2008) was performed in order to determine which were the variables that best explained most of the variability. It was carried out with the InfoStat software. The analysis was prepared using the averages of each of the variables analyzed.

A Clustering was performed by UPGMA (Unweighted Pair Group Method with Arithmetic mean) (Garcia-Vallvé & Puigbo 2009). The results obtained were represented by a dendrogram, which showed the similarity relationships among the taxa. This analysis was performed with the PAST software (Hammer & Harper 2001) and the chosen similarity index was Bray-Curtis.

Results

For all the variables, the assumptions were not validated, even after transformation by Ln, so the Kruskal-Wallis non-parametric test was performed on the seven variables. The test carried out allowed showing statistically significant differences between the following species (P =< 0.05). For each of the variables analyzed, it is detailed whether the difference is greater (>) or less (<) and with respect to which other species.

Major Equatorial Diameter (MAED) (Fig. 1):
Ctenitis aspidioides > *C. deflexa*, *C. distans*, *C. eriocaulis*, *C. falciculata*, *C. paranaensis*, *C. submarginalis*;
Ctenitis nervata > *C. deflexa*, *C. distans*, *C. eriocaulis*, *C. submarginalis*;
Ctenitis bigarellae > *C. deflexa.*
Minor Equatorial Diameter (MIED) (Fig. 2):
Ctenitis deflexa < *C. anniesii*, *C. aspidoides*, *C. bigarellae*, *C. distans*, *C. eriocaulis*, *C. falciculata*, *C. nervata*, *C. paranaensis*, *C. submarginalis.*
Polar Diameter (PD) (Fig. 3):
Ctenitis aspidoides > *C. deflexa*, *C. distans*, *C. paranaensis*, *C. submarginalis*;
Ctenitis deflexa < *C. anniesii*, *C. bigarellae*, *C. eriocaulis*, *C. falciculata*, *C. nervata*.
Length of Laesura (LL) (Fig. 4):
Ctenitis eriocaulis < *C. anniesii*, *C. aspidioides*, *C. bigarrellae*, *C. distans*, *C. falciculata*, *C. nervata*, *C. paranaensis*, *C. submarginalis*;
Ctenitis deflexa < *C. aspidioides*, *C. nervata*;
Ctenitis aspidioides > *C. submarginalis.*
Height of Folds (HF) (Fig. 5):
Ctenitis falciculata, *C. paranaensis*, *C. submarginalis* < *C. aspidioides*, *C. bigarellae*, *C. eriocaulis*, *C. nervata*;
Ctenitis deflexa > *C. falciculata*, *C. submarginalis*;
Ctenitis anniesii < *C. bigarellae*, *C. eriocaulis*.
Width of Folds (WF) (Fig. 6):
Ctenitis falciculata < *C. bigarellae*, *C. eriocaulis*, *C. nervata*, *C. submarginalis*;

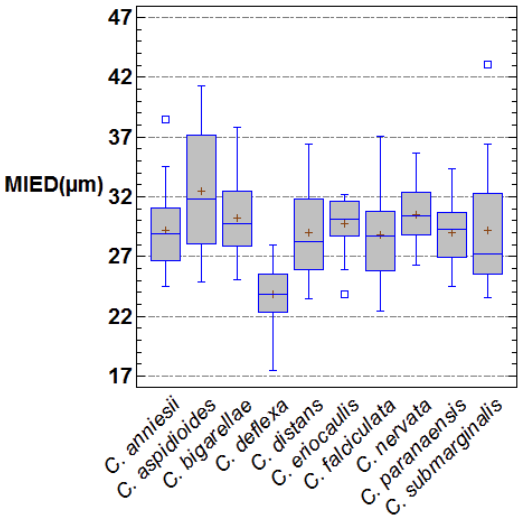

Figure 2 – Boxplots of the Minor Equatorial Diameter (MIED) for folded spores of *Ctenitis* species.

Ctenitis bigarellae > *C. anniesii*, *C. distans*, *C. paranaensis*;
Ctenitis nervata > *C. anniesii*, *C. aspidioides*, *C. deflexa*, *C. distans*, *C. paranaensis*.
Length of Folds (LF) (Fig. 7):
Ctenitis aspidioides, *C. nervata* > *C. anniesii*, *C. deflexa*, *C. eriocaulis*, *C. falciculata*, *C. submarginalis*;
Ctenitis submarginalis < *C. bigarellae*, *C. distans*, *C. paranaensis*;
Ctenitis bigarellae > *C. eriocaulis*.

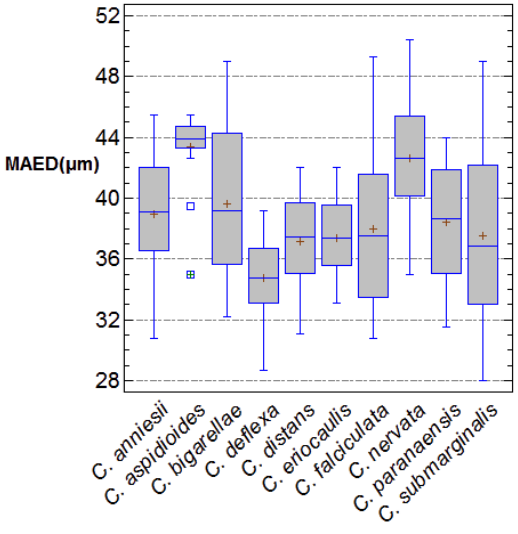

Figure 1 – Boxplots of the Major Equatorial Diameter (MAED) for folded spores of *Ctenitis* species.

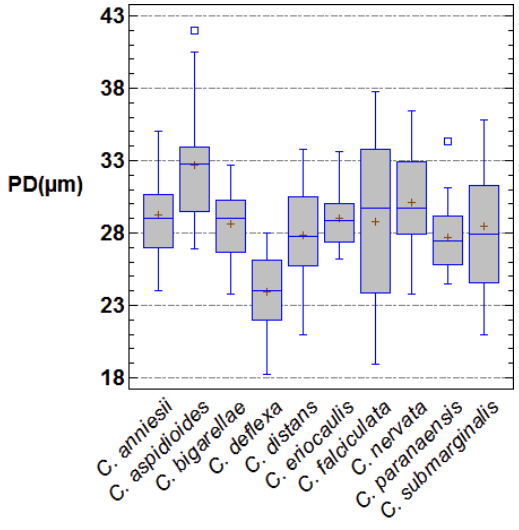

Figure 3 – Boxplots of the Polar Diameter (PD) for folded spores of *Ctenitis* species.

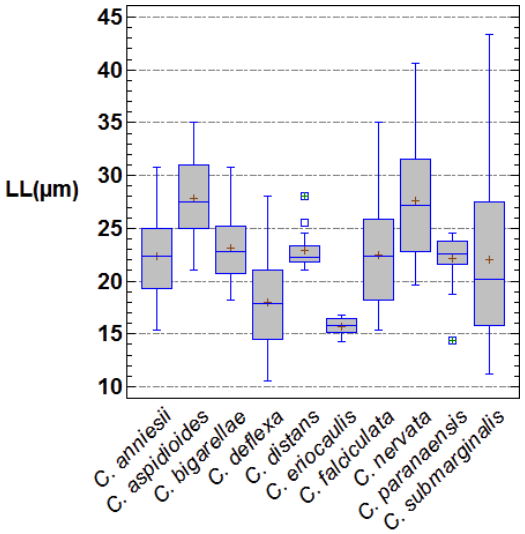

Figure 4 – Boxplots of the Length of Laesura (LL) for folded spores of *Ctenitis* species.

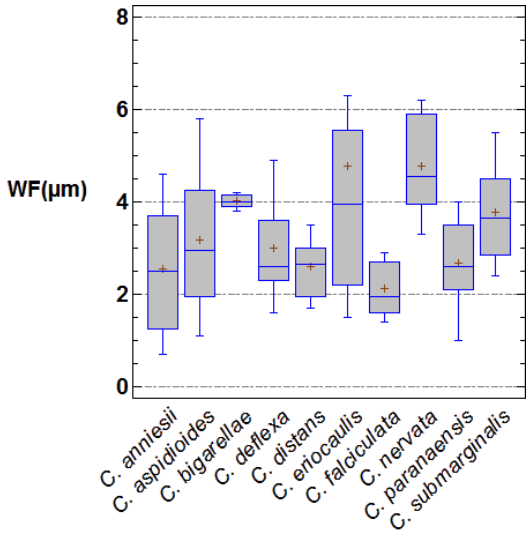

Figure 6 – Boxplots of the Width of Folds (WF) for folded spores of *Ctenitis* species.

A correlation PCA (Fig. 8) was performed to detect selectively taxonomic significant qualitative characters. Two dimensions scatterplot were constructed based on PCA outputs. According to the morphometry of the folds of each of the species, 4 groups were formed. These are showed by different coloration.

UPGMA cluster analysis was also useful in demonstrating distinct group of taxa. The ten species were grouped into 4 clusters (Fig. 9). The first (A: red circle) made up of *Ctenitis aspidioides* and *C. nervata*, the second (B: black circle) made up of *C. eriocaulis*, *C. submarginalis* and *C. bigarellae*, the third (C: green circle) made up of *C. distans*, *C. paranaensis*, *C. falciculata* and *C. anniesii* and the fourth and last (D: yellow circle) is made up of *C. deflexa*.

The average measurements of the folded spores of the *Ctenitis* species analyzed are shown in Table 1. The spores are monolete and light to dark brown color (Figs. 10-11).

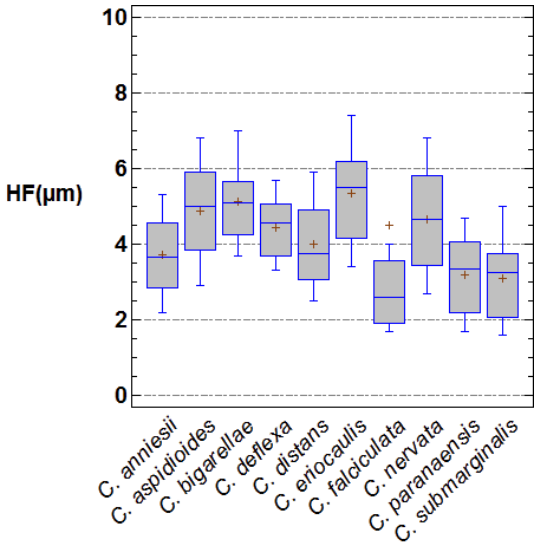

Figure 5 – Boxplots of the Height of Folds (HF) for folded spores of *Ctenitis* species.

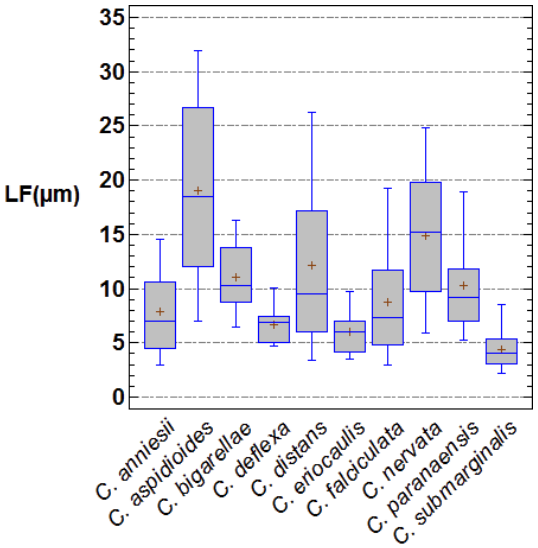

Figure 7 – Boxplots of the Length of Folds (LF) for folded spores of *Ctenitis* species.

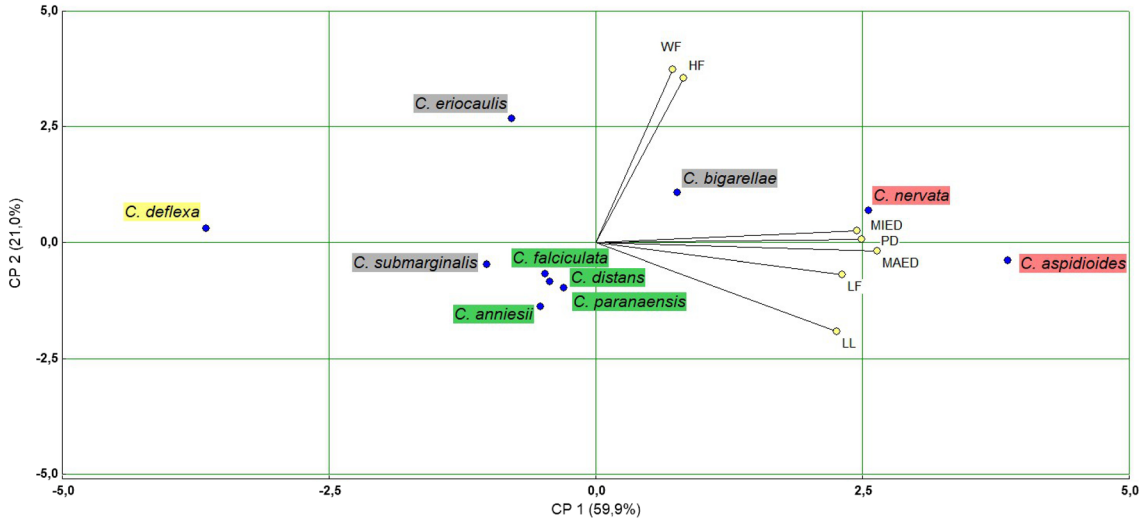

Figure 8 – Principal Components Analysis (PCA) performed with measurements for folded spores of *Ctenitis* species.

Discussion

The results obtained were on the same line with what was described by Viveros *et al.* (2018) and Gorrer *et al.* (2022). Complementary to these studies, a series of relatively unexplored quantitative characters in spores, in general, and in the *Ctenitis* genus, in particular, such as length, width and height of the folds, are here introduced. These characters provide new information and allow us to infer interspecific relationships, supported for others sporophyte characteristics. For example, *C. aspidioides* and *C. nervata* present the longest measurements (MAED, LF) in terms of their spores. They are also grouped by sporophyte characteristics, as mentioned by Viveros *et al.* (2018), having a pinnae with a 1/4–2/3 incision between the vertex of the segment and the coast. *C. deflexa*, on the other hand, has turned out to be the only species that has been significantly inferior in MAED, MIED and PD with respect to almost all the analyzed species of the genus. It is worth mentioning that it is also the only one that has a short creeping rhizome with scales that have many fimbriae (Viveros *et al.* 2018).

Other examples are the sporophytes of *Ctenitis bigarellae*, *C. falciculata*, *C. paranaensis* and *C. submarginalis* which are morphologically similar (Viveros *et al.* 2018). However, the data presented by Gorrer *et al.* (2022), together with the data presented here, show some differences among the spores. *C. bigarellae* and *C. submarginalis* have spores with short, subglobose and inflated folds but they are differentiated by color. The first one is dark brown and the latter is light brown. Characteristics those are reliable, unless there are hybridization problems, abortions or problems in sporogenesis (Wagner Jr. & Chen 1965), things that have not been recorded here for the mentioned species. On the other hand, C. *falciculata* and *C paranaensis* have light brown spores and their folds lack regularity - they are more dispersed, more elongated and narrower. Furthermore, *C. bigarellae* presents statistically higher folds than any of these

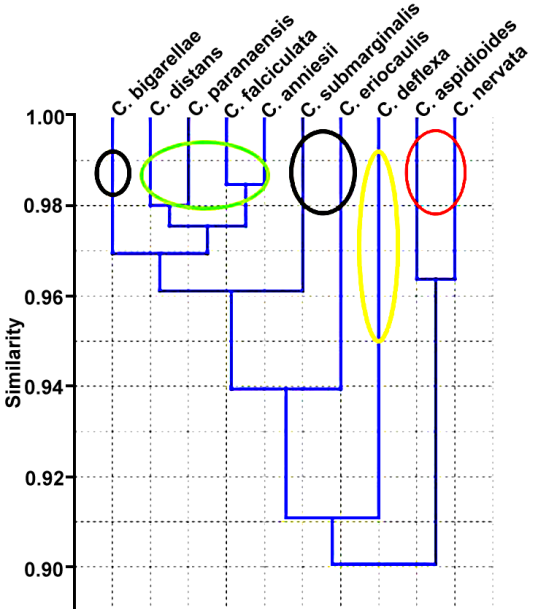

Figure 9 – Cluster analysis for folded spores of *Ctenitis*.

Table 1 – Average measurements, in µm, of the variables analyzed in the folded spores of *Ctenitis*.

Species	MAED	MIED	PD	LL	HF	WF	LF
Ctenitis anniesii	38.96	29.21	29.22	22.37	3.71	2.56	7.84
Ctenitis aspidioides	43.41	32.49	32.66	27.83	4.86	3.17	19.07
Ctenitis bigarellae	39.64	30.18	28.64	23.16	5.12	4.02	11
Ctenitis deflexa	34.72	23.88	23.9	17.96	4.44	3.01	6.69
Ctenitis distans	37.17	29	27.84	22.87	4.01	2.61	12.1
Ctenitis eriocaulis	37.4	29.77	29.03	15.72	5.36	4.77	6.07
Ctenitis falciculata	37.95	28.81	28.76	22.48	4.49	2.12	8.72
Ctenitis nervata	42.63	30.55	30.1	27.61	4.65	4.78	14.83
Ctenitis paranaensis	39	29	27.72	22.13	3.19	2.68	10.31
Ctenitis submarginalis	37.5	29.22	28.5	22.07	3.09	3.79	4.38

MAED = major equatorial diameter; MIED = minor equatorial diameter; PD = polar diameter; LL = length of laesura; HF = height of folds; WF = width of folds; LF = length of folds.

three species. *Ctenitis submarginalis* is a particular case, since its spores present a great variation in size, as mentioned by Tryon & Lugardon (1991) for Brazilian and Central American specimens. This species has been mentioned by Viveros *et al.* (2018) as highly variable regarding its morphology. Perhaps it can be considered a complex species. In addition, he pointed out that one of the reasons for this variation may be the difference of altitude at which it grows (50–1,600 masl). According to the records observed, it also presented enormous variability in the quantitative characters of its spores. It presented great amplitude in MAED, PD and LL, while its values were very restricted in terms of LF. On the contrary, *C. eriocaulis* and *C. deflexa* are the species with the lowest amplitude within the quantitative variables studied.

Adeonipekun *et al.* (2021) carried out a PCA with spores of ferns from Nigeria. In their analysis, they highlighted that the characters that best explained the variability in their spores are the PD, MAED and P/E ratio, with an accumulated variability of 95%. In contrast, in our study, the accumulated variability in axes 1 (60%) and 2 (21%) is 81%. The factors that are best located on axis 1 are MAED, MIED, PD and LF, while for axis 2 they are HF and WF. On the other hand, groupings were also found in the PCA, as Ramírez-Valencia *et al.* (2013) did with the spores of *Serpocaulon* species.

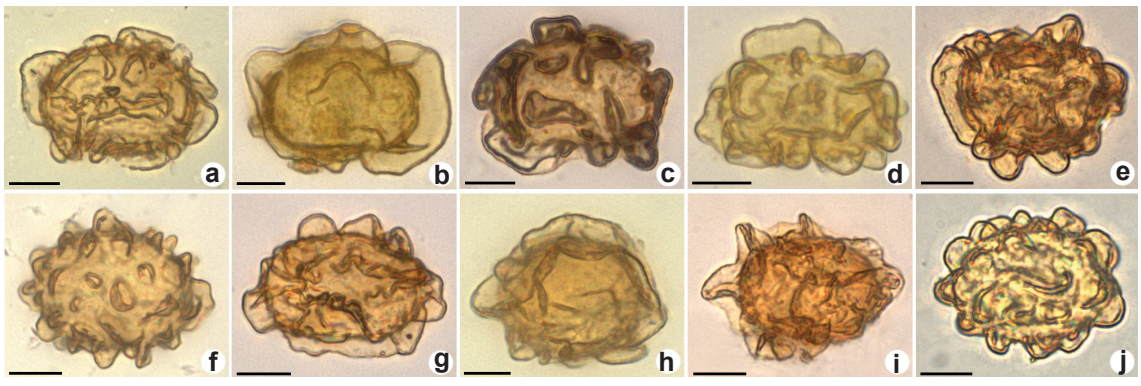

Figure 10 – a-j. Folded Spores of *Ctenitis* from Southern Cone of America with LM – a. *C. anniesii*; b. *C. aspidioides*; c. *C. bigarellae*; d. *C. deflexa*; e. *C. distans*; f. *C. eriocaulis*; g. *C. falciculata*; h. *C. nervata*; i. *C. paranaensis*; j. *C. submarginalis*. Scale bars: a-j = 10 µm.

Dendrograms support the evidence from both ANOVA and PCA. They also support the morphological analysis, since it allows for the visualization of some groupings, represented by the same coloration in both the PCA and the dendrogram. On the one hand we see *C. deflexa* whose morphometry stands out for being significantly smaller than the other species. On the other hand are *C. aspidioides* and *C. nervata*, which have large measurements and folds significantly longer than the rest. On third the group of *C. bigarellae*, *C. eriocaulis* and *C. submarginalis* that present subglobose and inflated and more or less regular folds. And finally, the other species (*C. distans*, *C. paranaensis*, *C. falciculata and C. anniesii*) that they present short and subglobose but irregular folds, with intermediate morphometric characters.

Within the folded spores species of *Ctenitis* bearing from Southern Cone of America, it is difficult to group or differentiate sets of species. However, the morphometric characters analyzed here help us to establish groups species based mainly on color, equatorial diameters and the length of their folds.

The species that showed statistically significant differences in terms of the major equatorial diameter (MAED) are *C. aspidioides and C. nervata*. The species with the lowest amplitude within the variable was *C. aspidioides*, while the one with the greatest internal amplitude was *C. submarginalis*. In the minor equatorial diameter (MIED), *C. deflexa* was the species with significantly lower measurements compared to the rest of the analyzed species.

The polar diameter (PD) showed that the species with the highest statistically significant differences is *C. aspidioides* .On the other hand, *C. deflexa* is the species with significantly the lowest values.

As for the length of laesura (LL), *C. eriocaulis* has significantly lower measurements.

Ctenitis falciculata, *C. paranaensis* and *C. submarginalis* are the species with the lowest folds (HF). Regarding the width of the folds (WF), *C. bigarellae* and *C. nervata* are the species with the highest values, while *C. falciculta* has the lowest. Finally, *C. aspidioides* and *C. nervata* present significantly longer fold values (LF).

The PD and MIED have been the least variable characteristics within the genus.

On the contrary, laesura is the variable with the greatest internal amplitude among the species analyzed. Thus, the statistical study carried out on these species of the genus *Ctenitis* allowed us to relate some morphological characteristics of the sporophyte with the morphology of their respective spores, which also allows us to infer that these quantitative variables could be taken into account when carrying out phylogenetic analyzes in the studied genus or related groups.

Gathering all the analyzed variables, four spore sets are proposed for the spores of the genus *Ctenitis*. The "elongate-type" is made up of *C. aspidioides* and *C. nervata*, which are the species with the longest measurements. The "medium-type" is made up of *C. anniesii*, *C. distans*, *C. falciculata* and *C. paranaensis* which are the ones with intermediate measurements. The "handle shape-type" is represented by *C. bigarellae*, C. *eriocaulis* and *C. submarginalis*. Finally, the

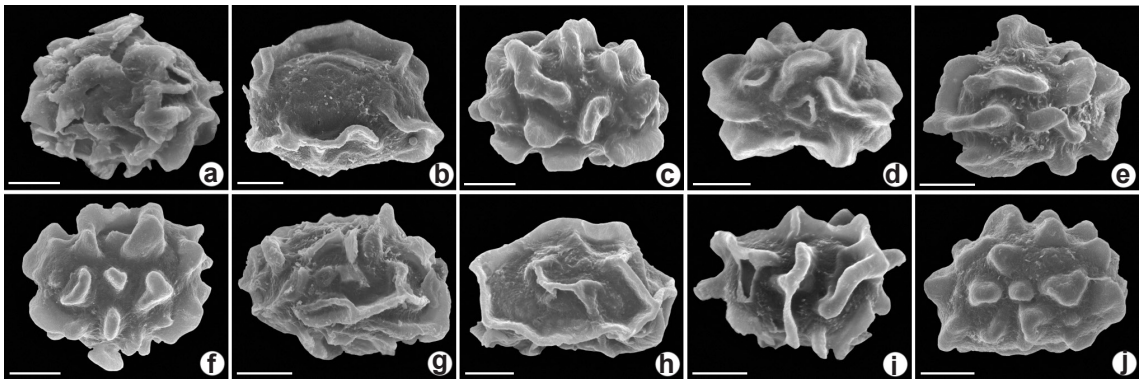

Figure 11 – a-j. Folded Spores of *Ctenitis* from Southern Cone of America with SEM – a. *C. anniesii*; b. *C. aspidioides*; c. *C. bigarellae*; d. *C. deflexa*; e. *C. distans*; f. *C. eriocaulis*; g. *C. falciculata*; h. *C. nervata*; i. *C. paranaensis*; j. *C. submarginalis*. Scale bars: a-j = 10 μm.

"short-type", consists only of *C. deflexa* which is the species that registers several significantly smaller measurements.

The morphological and morphometric data of spores together with the statistical analysis provided useful information for the distinction of *C. deflexa* and some groups of species within the genus *Ctenitis* in the Southern Cone of America. These results can greatly contribute to the palynological studies of ferns and the studies of aeropalynological and palaeopalynological characters. Besides, they can shed light on the taxonomic characters of the genus and can be used in future phylogenetic studies.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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