

Ferns and Lycophytes as new challenges

A new record of green spore in *Lomaria* (Blechnaceae, Polypodiopsida)

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Abstract

Fern spores play a vital role in dispersal. The unique features of spores, such as size, thickness, and ornamentation can aid in taxonomic decisions and division of groups. Moreover, certain species of ferns possess green spores containing chlorophyll, an uncommon feature that can reinforce the evidence of relatedness within a specific group. *Lomaria spannagelii* is a Neotropical species endemic to Brazil, and, even though green spores have been reported in its sister species, *Lomaria nuda* and *Lomaria discolor*, the presence of these spores has not yet been investigated in *L. spannagelii*. In this study, we investigated the morphology of *L. spannagelii* spores and sporangia, and, additionally, we determined the chromosome count of the species. Our results showed that the analyzed species has green spores, a characteristic shared with *L. nuda* and *L. discolor*, and has $2n = 64$ chromosomes. This discovery provides new insights into this fern genus and emphasizes the importance of spore color and chromosome count in systematic and evolutionary studies of ferns.

Key words: *Blechnum*, chlorophyllous spore, chromosome count, ferns.

Resumo

Os esporos de samambaias desempenham um papel vital na dispersão. As características únicas dos esporos, como tamanho, espessura e ornamentação, podem auxiliar nas decisões taxonômicas e na divisão dos grupos. Além disso, certas espécies de samambaias possuem esporos verdes contendo clorofila, característica rara que pode reforçar a evidência de parentesco dentro de um grupo específico. *Lomaria spannagelii* é uma espécie Neotropical endêmica do Brasil e, embora esporos verdes tenham sido relatados em suas espécies irmãs, *Lomaria nuda* e *Lomaria discolor*, a presença desses esporos ainda não foi investigada em *L. spannagelii*. Neste estudo, investigamos a morfologia dos esporos e esporos de *L. spannagelii* e, adicionalmente, determinamos a contagem cromossômica da espécie. Nossos resultados mostraram que a espécie analisada possui esporos verdes, característica compartilhada com *L. nuda* e *L. discolor*, e possui $2n = 64$ cromossomos. Esta descoberta fornece novos insights sobre o gênero e enfatiza a importância da cor dos esporos e contagem de cromossomos em estudos taxonômicos e evolutivos de samambaias.

Palavras-chave: *Blechnum*, esporo clorofilado, contagem cromossômica, samambaias.

Introduction

Ferns are a diverse group of plants that can vary widely in their morphological and physiological characteristics, including their spores. Fern spores are the dispersal unity in this group of vascular plants, which is mostly disseminated by wind (Schneider 2013). This seedless group

produces spores in specialized structures called sporangia, and two types are recognized: the eusporangium and leptosporangium, with the latter being a synapomorphy of Polypodiidae (PPG I 2016). Most fern species are homosporous, *i.e.*, produce a unique type of spore (in size and surface ornamentation) capable of germinating

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into a hermaphroditic gametophyte. However, few families or genera are heterosporous, *i.e.*, two types of spores are produced, the microspore and the megaspore (different by size and surface ornamentation). Spores play an important role in taxonomic decisions, as they are used to identify genera or segregate species based on their size, shape, and surface features (Moran *et al.* 2018; Silva *et al.* 2019; Pereira & Prado 2022).

Another spore characteristic that can be observed is their color. The majority of fern species have brown achlorophyll spores, however, in some families or genera, the spores are green (Tryon & Lugardon 1991). Green spores are characterized by the presence of chlorophyll, making them easily recognizable under light microscopy. However, there are instances where the chlorophyll is not evident in light microscopy, even though being present, characterizing the cryptochlorophyllous spores (Sundue *et al.* 2011; Tseng *et al.* 2017). In a study that analyzed 2,068 fern species, Mellado-Mansilla *et al.* (2021) found that 441 species (17%) possess chlorophyllous spores, while only 1% (30 species) have the cryptochlorophyllous type, indicating the rarity of the latter.

Spores size has been observed to exhibit a correlation with chromosome number, serving as an indicator of polyploid levels (*e.g.*, Pereira 2015; Barrington *et al.* 2020), being a whole-genome duplication or an unreduced spore ($n = 2n$) responsible for a polyploid species (Grusz *et al.* 2021). Chromosome counting is an important mechanism in understanding the evolutionary process, especially in ferns, where significant numbers of chromosomes can be found, demonstrating polyploidy as a main mechanism in the evolutionary process (*e.g.*, Marcon *et al.* 2003).

Considering the importance of spores for taxonomy and species reproduction, and in light of the publication of the new Blechnaceae classification and the revision of species from Brazil, we were questioning if green spores could be a synapomorphy of the *Lomaria* genus. In such a manner, we conducted a study on the spores of *Lomaria spannagelii* (Rosenst.) Gasper & Dittrich, an endemic species of southern and southeastern Brazil within the cold regions of the Atlantic Forest (Dittrich *et al.* 2018). Our objectives were to determine whether this species possesses green spores, similar to *Lomaria nuda* (Labil.) Willd. and *Lomaria discolor* (G. Forst) Willd. (Sundue & Rothfels 2014), and to characterize the morphology of the spores and sporangia of *L. spannagelii*.

Additionally, we aimed to provide the chromosome count for this species, as this information was absent from the existing literature.

Material and Methods

Samples of fresh spores of *Lomaria spannagelii* from Urubici, Santa Catarina, southern Brazil, were collected in February 2021 (R.B. Sühs 20210001 FURB 67232) for spore analysis. Also, from the same locality, one individual was collected and kept at the Universidade Regional de Blumenau greenhouse to obtain young root tips for posterior chromosome count.

We used a Zeiss Axiostar Plus microscope to perform the light microscopy analysis of 54 fresh spores and 36 sporangia. Then, the images were edited and measured using ImageJ (Abràmoff *et al.* 2004). Measurements of fresh spores were made for polar and equatorial diameters. Additionally, we measured pedicel and counted annulus cells of the sporangia, from dried exsiccate deposited under the following registration numbers: *A. Salino* 14788 FURB 35526, *J.L. Schmitt* 3169 FURB 35038, *E. Caglioni* 303 FURB 39717, *R. Wasum s.n.* FURB 44317, *A.L. de Gasper* 3099 FURB 41127, *L.A. Funez* 8760 FURB 64371, *A. Kassner-Filho* 6269 FURB 67820, *R. Reitz* 10646 FURB70278. The analyzed spores are from individuals from Rio Grande do Sul and Santa Catarina, where the species is most abundant within the *Araucaria* Forest.

To determine the chromosome count, we followed the methodology described by Guerra & Souza (2002). Young root tips, from the cultivated specimen, were utilized to gather the meristematic tissue located below the tip of the root. This tissue is rich in cells that are actively dividing and densely packed. Before fixation, the roots underwent a four-hour pre-treatment at 18 °C with 8-Hydroxyquinoline to halt mitosis. Following this, the roots were fixed overnight in Carnoy's solution (3:1 ethanol-glacial acetic acid) and stored in the freezer until use.

To prepare the slides, the material was cleaned twice for five minutes in distilled water. Subsequently, it was put in hydrochloric acid for 20 minutes for hydrolysis and then was digested in an enzymatic solution containing 2% cellulase and 20% pectinase for one hour. The root cap was then excised using scalpels on a Stemi 508 Stereo Microscope, and the cells were carefully separated. Subsequently, the material was placed under a glass cover slip and subjected to pressure

to facilitate chromosome separation, followed by removal in liquid nitrogen. Finally, the slide was treated with Giemsa's staining solution. This process was repeated until a clear chromosome count was obtained.

Results

The spores of *Lomaria spannagelii* are monolete and green, with $21.71 \pm 1.35 \mu\text{m}$ in polar diameter and $27.0 \pm 1.67 \mu\text{m}$ in equatorial diameter (Fig. 1a). The pedicel of the sporangia is $434.55 \pm 155.14 \mu\text{m}$ long, and the annulus is composed of 24 cells (Fig. 1b). While capturing the images, we observed that the number of spores differed among sporangia, with some containing 16, some with 32, and some with 64 spores (Fig. 1c-e). However, we were unable to determine if all spores were viable, although the spores have the same apparent size. The results obtained through the mitotic cells reveal

$2n = 64$ chromosomes, with $3.14 \pm 0.51 \mu\text{m}$ long and with a rod shape (Fig. 2).

Discussion

Our findings have confirmed that *L. spannagelii*, similar to its sister species *L. nuda*, possesses green spores. In a phylogenetic analysis of four out of the six species of the genus, Gasper *et al.* (2016) identified two clades of *Lomaria*, both containing species with green spores, suggesting that this may be a shared trait of the genus. The genus *Lomaria* was first described in 1809 (Willdenow 1809) and has been recently recognized in a new classification of ferns and lycophytes (PPG I 2016). This genus is characterized by deeply grooved rachises and discolorous blades (Gasper *et al.* 2016; Dittrich *et al.* 2018), and comprises only 2.2% of the family, with six species with a pantropical distribution.

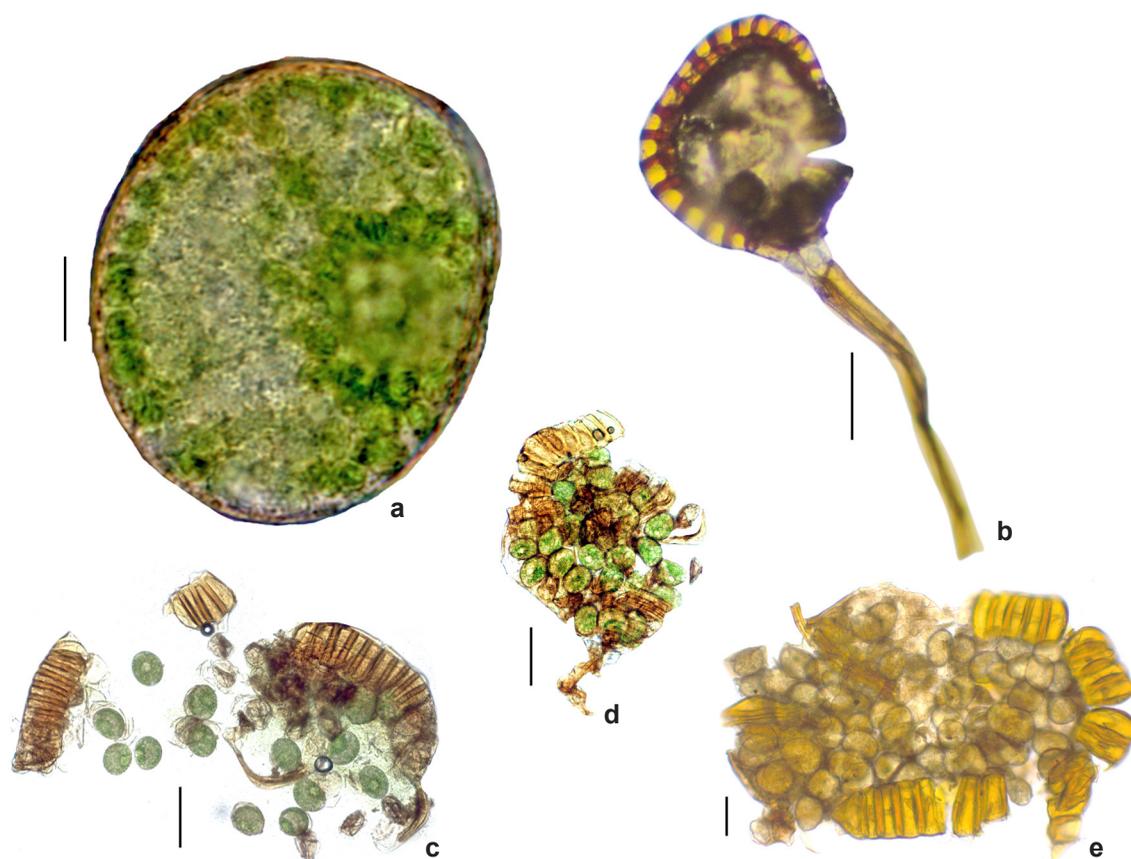


Figure 1 – a-b. Light microscopy details of *Lomaria spannagelii* – a. green spore; b. sporangium. c-e. Sporangia presenting distinct spore numbers under light microscopy – c. 16 spores; d. 32 spores; e. 64 spores. Scale bars: a = $100 \mu\text{m}$; b = $5 \mu\text{m}$; c, d, e = $50 \mu\text{m}$. (a-d. R.B. Siihs 20210001 FURB 67232; e. A. Kassner-Filho 6269 FURB 67820).

Characters of spores are widely used as evidence to prove the significance of phylogenies and the proximity of clades, and the same applies to the Blechnaceae family (Moran *et al.* 2018). Within Blechnaceae, spores exhibit a great variation, having a wide range of characteristics. For example, in the subfamily Stenochlaenoideae, the exine takes on a contoured form with tubercles or short ridges. Tortuous filaments can be seen in *Parablechnum*, narrow folds and granulate deposits in *Lomaridium*, fibrillose or foliaceous perine in *Sadleria*, and a non-folded smooth or granulate perine in *Austroblechnum* (Moran *et al.* 2018). In *Lomaria*, on the other hand, the spores differ by their folded perine (the outer layer of the spore), with smooth to granulate microstructures on its surface (Moran *et al.* 2018). As a widely dispersed genus, *Lomaria* may vary in spore sizes among its species. This variation also occurs inside the species, and studies that have analyzed *L. spannagelii* spores have shown a range of 40 to 72 μm in equatorial diameter, and 27 to 42 μm in polar diameter (Passarelli *et al.* 2010; Silva *et al.* 2019).

Green spores, distinctly from the achlorophyllous non-green spores, are considered to have short-lived viability, with germination occurring in less than three days after sowing (Lloyd & Klekowski Jr. 1970). This spore type is prevalent in a few families, with the majority, if not all, of the species having green spores. Examples

of families with green spores include Equisetaceae, Osmundaceae, and Onocleaceae (Lloyd & Klekowski Jr. 1970). Among them, Onocleaceae is a sister family of Blechnaceae (PPG I 2016; Gasper *et al.* 2017) and, until now, there have been only two records of green spores in Blechnaceae, both in *Lomaria*, specifically in *L. nuda* (Stone 1961) and in *L. discolor* (Sundue & Rothfels 2014).

Approximately 70% of fern species that produce chlorophyllous spores grow as epiphytes, making up almost 37% of all epiphytic fern species. The most common families for these ferns are Polypodiaceae (subfamily Grammitioideae) and Hymenophyllaceae, with a collective representation of over 100 species reported to have green spores (Mellado-Mansilla *et al.* 2022). Fern species with chlorophyllous spores, which constitute almost 60% of those present in waterlogged soils, are overrepresented in these environments. (Mellado-Mansilla *et al.* 2022). *Lomaria spannagelii*, a terrestrial fern species commonly found in *Araucaria* forests and areas with flooded soils, is particularly abundant along streams at higher altitudes (Dittrich *et al.* 2018).

Concerning the analysis of chromosome numbers, the results may provide valuable insights into the evolutionary relationships of the species under investigation. Our study revealed a chromosome count of $n = 32$ for *L. spannagelii*. This finding is significant when compared to a

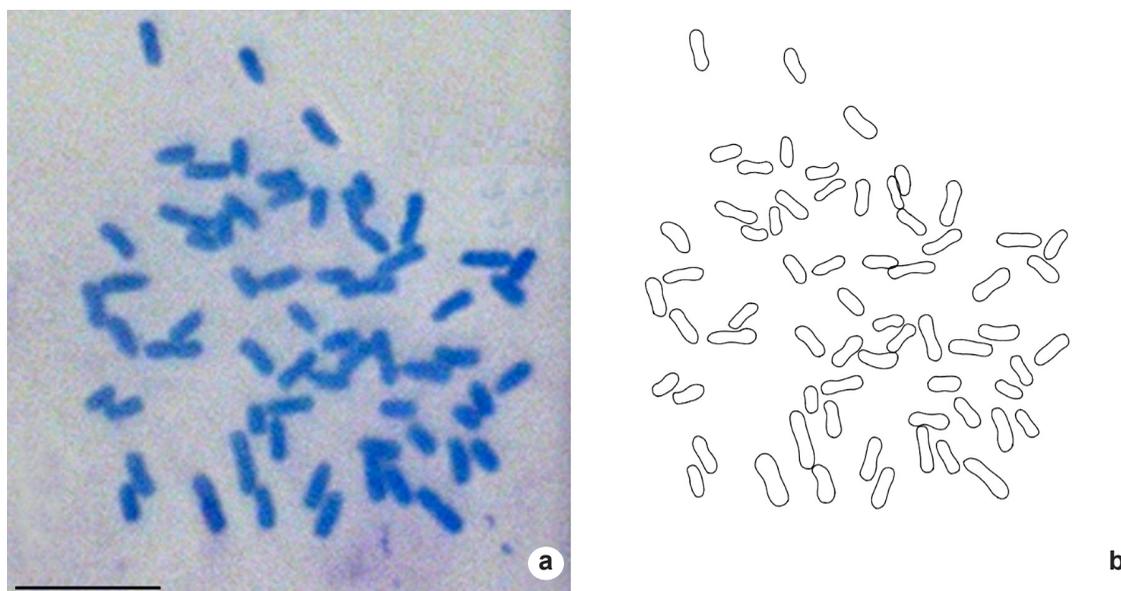


Figure 2 – a-b. Chromosome count of *Lomaria spannagelii* – a. mitotic metaphase, $2n = 64$; b. graphic representation on an equal scale. Scale bar = 10 μm . (Analyzed material R.B. Sühs 20210001 FURB 67232).

previous study that analyzed spores of its sister species, *L. nuda*, which had a chromosome number of $n = 28$ (Löve *et al.* 1977). Additionally, it is noteworthy to consider the chromosomal variations present within the Blechnaceae family. *Lomaridium ensiforme* (Liebm.) Gasper & Dittrich, a species from a closely related genus, shares the chromosome number of $n = 28$ with *L. nuda* (Smith & Foster 1984). Conversely, the genus *Icarus*, which belongs to the same clade, exhibits a chromosome number of $n = 33$ (Rice *et al.* 2015).

Previous studies have investigated the variation of chromosome numbers in other species belonging to this family, revealing the common occurrence of specific chromosomal variations, such as the variation observed in *Blechnum occidentale* L., with an established number of $2n = 124$ chromosomes [$n = 31$; Marcon *et al.* (2003), $2n = 186$ (González *et al.* 2016)], revealing polyploid events. These variances highlight the diverse chromosomal count within and between genera, shedding light on the evolutionary dynamics within the family. In the context of *Lomaria*, the absence of documented polyploids prompts an examination of the underlying causes for the observed variation in chromosome number. It is plausible to attribute this variation to potential events, such as aneuploidy and dysploidy, which give rise to subtle alterations in the chromosomal count (Levin 2002). Aneuploidy involves the loss or gain of one or more chromosomes, resulting in an unbalanced genetic constitution with no evolutionary function (Guerra 2008). In contrast, dysploidy induces changes in chromosome number without disrupting the overall balance (Friebe *et al.* 2005). Another explanation would be the presence of B chromosomes, which are extra chromosomes beyond the usual set that can deviate from typical Mendelian segregation patterns and often exhibit preferential inheritance (Houben 2017).

Acknowledgements

The authors thank R.B. Sühs, for collecting samples. ALG thanks to CNPq, for the productivity grant (311303/2020-0) and Fapesc nº 29/2021, for the new equipment that allowed us to obtain better images.

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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