Rodriguésia 75: e00882023. 2024
http://rodriguesia.jbrj.gov.br
DOI: http://dx.doi.org/10.1590/2175-7860202475011

Plant anatomy: history and future directions

Characterization and evolution of the aerenchyma diaphragm of the stem in *Eleocharis* (Cyperaceae)

Jaqueline Priscila Bispo de Almeida Cardoso Marcondes[1,4], *Rafael Trevisan*[2], *Maria Cecília de Chiara Moço*[3], *Giovanna Wisniewski Peletti*[1,5] & *Cleusa Bona*[1,6,7]

Abstract

Aerenchyma is a characteristic tissue of aquatic plants, characterized by the presence of air lacunae commonly septated by diaphragms. These are formed by one or more layers of stellate cells, which allow for the passage of gases. Most species of *Eleocharis* grow in wet or flooded soils and have aerenchyma in their aerial stems. However, extensive studies on this structure, which could contribute to ecological and phylogenetic studies of the group, are lacking. This work describes the structure of the diaphragm in the stem of *Eleocharis* species and investigates the evolution of this characteristic in the genus. Fifty-three species were analyzed under light and scanning electron microscopy. We analyzed the evolution of the characteristics by reconstructing their ancestral states based on the previously published original phylogeny. The diaphragms in *Eleocharis* vary mainly in the number of layers, cell shape, and cell wall thickness. The typical diaphragm of the genus is composed of three to four layers of stellate cells, with microprojections and secretory cells. The diaphragm of the group's ancestor had practically the same characteristics as the genus's typical diaphragm.
Key words: anatomy, aquatic macrophytes, evolution, micromorphology, stellate cells.

Resumo

Aerênquima é um tecido comum nas plantas aquáticas, caracterizado pela presença de lacunas de ar comumente septadas por diafragmas. Esses, são formados por uma ou mais camadas de células, geralmente braciformes, que permitem a passagem de gases. A maioria das espécies de *Eleocharis* cresce em solos úmidos ou inundados e apresenta aerênquima no caule. No entanto, essa estrutura carece de estudos amplos que poderiam contribuir aos estudos ecológicos e filogenéticos do grupo. Este trabalho descreve a estrutura do diafragma no caule de espécies de *Eleocharis* e investiga a evolução desse caractere no gênero. 53 espécies foram analisadas em microscopia de luz e eletrônica de varredura através de secções transversais e longitudinais do caule. A análise da evolução dos caracteres foi feita pela reconstrução de estado ancestral, baseada na filogenia original anteriormente publicada. Os diafragmas em *Eleocharis* variam principalmente em número de camadas, formato das células e espessura da parede celular. O diafragma típico do gênero é composto de três a quatro camadas de células braciformes lobadas, com microprojeções e células secretoras. O diafragma do ancestral do grupo apresentava praticamente as mesmas características do diafragma típico para o gênero.
Palavras-chave: anatomia, macrófitas aquáticas, evolução, micromorfologia, célula braciforme.

[1] Federal University of Paraná, Sector of Biological Sciences, Department of Botany, Laboratory of Structural Botany, Jardim das Américas, Curitiba, PR, Brazil.

[2] Federal University of Santa Catarina, Center of Biological Sciences, Department of Botany, Laboratory of Plant Systematics, Trindade, Florianópolis, SC, Brazil. ORCID: <https://orcid.org/0000-0003-4145-8299>.

[3] Federal University of Rio Grande do Sul, Department of Botany, Laboratory of Plant Anatomy, Porto Alegre, RS, Brazil. ORCID: <https://orcid.org/0000-0001-9478-8713>.

[4] ORCID: <https://orcid.org/0000-0002-1223-8396>.

[5] ORCID: <https://orcid.org/0009-0003-4861-866X>.

[6] ORCID: <https://orcid.org/0000-0003-4527-8236>.

[7] Author for correspondence: bonacleusa@gmail.com

Introduction

Aerenchyma is a typical tissue in aquatic plants, characterized by large air spaces between the cells. Its main function is to mitigate the lack of oxygen in flooded regions (Justin & Armstrong 1987); however, it also contributes to the circulation of CO_2 during photosynthesis (Constable *et al.* 1992; Li & Jones 1995) and the circulation of hormones such as ethylene (He *et al*. 1996). The aerenchyma forms both due to cellular lysis and without it (Seago *et al*. 2005). Commonly, the aerenchyma that forms without cell lysis has large gaps septated by diaphragms.

The diaphragms are formed by one or more layers of cells that delimit the gaps, maintain intercellular spaces, and play an important role in maintaining structure and metabolic balance (Sculthorpe 1967). These structures allow for the continuous flow of gases, provide resistance and mechanical stabilization to the organ, restrict water entry when insect damage occurs, and provide a lateral transport pathway through the cortex, allowing for the storage of substances such as tannins and starches (Snow 1914; Sculthorpe 1967; Kaul 1971, 1972; Dickison 2000). Armstrong (1979) adds that wall thickness, porosity (intercellular spaces), and the distribution of diaphragms also influence the circulation of gases.

Eleocharis species have aerial upright stems that originate from a congested, rhizomatous, or stoloniferous stem (Trevisan & Boldrini 2008). The upright stems are leafless and photosynthetic. Due to the small size and simplicity of the plant, only a few morphological features are available for phylogenetic analyses that may aid in infrageneric classification. The use of anatomical characteristics complements the morphological and molecular analyses, helping to clarify the relationships between the subgenera (Hinchliff & Roalson 2009).

Despite these efforts, the phylogenetic studies of *Eleocharis* show that the most recent classification by González-Elizondo & Peterson (1997) does not reflect the phylogenetic relationships within the group (Roalson & Friar 2000; Yano *et al*. 2004; Roalson *et al*. 2010). According to Roalson *et al*. (2010), most of the seven clades that group the species of *Eleocharis* together are well supported, but some groups still show variations in their positions in the analyses, mainly those of cosmopolitan distributions. For this reason, the previously cited authors emphasize that a satisfactory classification that delimits these groups has not yet been found and that future research in search of new characters for analysis is therefore necessary. It is known that most species of *Eleocharis* preferentially grow in environments with wet or flooded soils and can be found either totally or partially submerged (Trevisan & Boldrini 2008). Due to this plasticity in the environmental conditions needed for survival and reproduction, many emerging or submerged species present with highly developed aerenchyma tissue, both in the root and in the stem (Marcondes *et al*. 2021).

The stem aerenchyma in *Eleocharis* is composed of air cavities, which are often septated by diaphragms (Metcalfe 1971; Marcondes *et al*. 2021). According to the distribution of the cavities, two types of stem architecture are found in the subgenus *Limnochloa* species: spongy and septated (Hinchliff & Roalson 2009). The spongy stem presents with several air columns in the central region of the organ, intersected transversally by diaphragms, while the septated stem has a central air column, which occupies most of the organ and is interrupted by large, transverse diaphragms (Marcondes *et al*. 2021). These two patterns were recorded in 68 *Eleocharis* species, wherein spongy was the more frequent and was subdivided into two types by Marcondes *et al*. (2021).

Studies on the shape and anatomy of the stem have not detailed the structures of the diaphragms, which represent a potential source of characteristics that could help delimit the clades, subclades, and even problematic species of the genus. Since the stem in *Eleocharis* is the main photosynthetic organ and consists predominantly of aerenchyma with diaphragms, we believe that this tissue can provide morphological variations that could help in the characterization of the groups, as well as help us to understand the phylogenetic relationships of the genus. Thus, in the present, we aim to describe the anatomy and micromorphology of the diaphragms present in the aerial stem aerenchyma in representatives of four of the seven phylogenetic clades indicated for this genus (Roalson *et al*. 2010) to verify the existence of anatomical characteristics exclusive to each clade, and to describe the evolutionary history of the characteristics of the genus.

Material and Methods

We analyzed 53 species of *Eleocharis* (Tab. 1). When available, each species was represented by three samples from different locations and environments. The samples were obtained from fixed and herborized material. Species from four

Table 1 – *Eleocharis* (Cyperaceae) species used in the characterization of the diaphragms present in the stem aerenchyma.

Species	Voucher	Origin	Habitat
E. acicularis (L.) Roem. & Schult.	*Ahles, H. E. 84582* (UPCB)	U.S	_____
	Wallnöfer, B. 13753 (MBM)	Austria	Edge of dam on silting ground
	Lampinen, R. 17611 (MBM)	Finland	River bank, sandy soil. Amphibian
E. acuta R. Br.	*Lepschi, B. J. 1553* (MBM)	Australia	Edge of dam, sandy soil. Emergent
E. acutangula (Roxb.) Schult.	*La Yela s.n.* (MBM127313)	Argentina	Periodically flooded land
	Matthew, K. M. s.n. (MBM69447)	India	_____
	Stehmann, J. R. & Perdigão, G. 1705 (MBM)	MG, Brazil	Emergent
E. albida Torr.	*Brumbach, W. C. 9031* (MBM)	U.S	Edge of the pond
E. bonariensis Nees	*Pedersen, T. M. 10034* (MBM)	Argentina	Sandbanks on the river
	Hatschbach, G. et al. 78115 (MBM)	SC, Brazil	River edge
E. compressa Sull.	*Isaac, J. A. 9766* (MBM)	U.S	River edge
	Whitehouse, E. 15302A	U.S	Moist clayey soil
E. congesta D. Don	*Bai-Zhong X. 4018* (MBM)	China	_____
	Bai-Zhong X. 3753 (MBM)	China	_____
E. contracta Maury *ex* Micheli	*Pedersen, T. M. 10027* (MBM)	Argentina	Wet soil, on low ground
	Schinini, A. 5263 (MBM)	Argentina	River bank, open field
	Alvarez, D. et al. 10261 (MBM)	Mexico	Lowland
E. cylindrica Buckley	*Pedersen, T. M. 9093* (MBM)	Argentina	Moist clayey soil
	Pedersen, T. M. 9194 (MBM)	Argentina	Floodable soil
E. cylindrostachys Boeckeler	*Lepschi, B. J. 1435* (MBM)	Australia	River bed. Emergent
E. densicaespitosa R. Trevis. & Boldrini	*Bertels, A. 1043* (UPCB)	RS, Brazil	_____
	Krapovickas, A. & Cristobal, C. L. 20832 (MBM)	Argentina	On stream rocks
	Valduga, E. 161 (MBM)	RS, Brazil	_____
E. dombeyana Kunth	*Guaglianone, L. D. et al. 1410* (MBM)	Argentina	_____
	Pedersen, T. M. 11781 (MBM)	Argentina	Lagoon on the river bank
	Asplund, E. 7165 (MBM)	Ecuador	Lake edge. Amphibian
E. dunensis Kük.	*Pedersen, T. M. 15872* (MBM)	Uruguay	Wet soil
	Carnevali, R. 2316 (MBM)	Argentina	Wet Field

Species	Voucher	Origin	Habitat
E. elegans (Kunth) Roem. & Schult.	*Barbosa. E. & Silva, J. M. 1587* (MBM)	MS, Brazil	Flooded land (wetland)
	Balslev, H. & Madsen, E. 10442 (MBM)	Ecuador	River edge
	*Pedersen, T. M. 9663 (*MBM)	Argentina	Flooded channel margin
E. endounifascis Hinchliff & Roalson	*Marinho, A. M. 77* (MBM)	RN, Brazil	_____
E. engelmannii Steud.	*Ahles, H. E. 85429* (UPCB)	U.S	Low depression
	Kral, R. 39241 (MBM)	U.S	Low sandy field
E. equisetoides (Elliott) Torr.	*Kral, R. 43089* (MBM)	U.S	_____
E. erythropoda Steud.	*Morency, M. 917* (MBM)	Canada	Flooded channel margin
E. exigua (Kunth) Roem. & Schult.	*Bona, C. & Costa, D. 355* (UPCB)	PR, Brazil	——
E. filiculmis Kunth	*Pedersen, T. M. 9499* (MBM)	Paraguay	Marsh
	Davidse, G. 3774 (MBM)	Venezuela	Wet soil
	Irwin, H. S. 31610 (MBM)	BA, Brazil	Moist sandy soil
E. flavescens (Poir.) Urb.	*Whitehouse, E. 17624* (MBM)	U.S	_____
	Hatshbach, G. et al. 74110 (MBM)	MS, Brazil	_____
	Bona, C. & Costa, D. R. 340 (UPCB)	PR, Brazil	Flooded land. Emergent
E. geniculata (L.) Roem. & Schult.	*Berghen, C. V. 6346* (MBM)	Senegal	_____
	Bona, C. et al. 495 (UPCB)	PR, Brazil	_____
	Pedersen, T. M. 6949 (MBM)	Argentina	Moist clayey soil
E. hatschbachii R. Trevis.	*Krapovickas, A. et al. 29519* (MBM)	Argentina	_____
	Gibbs, P. E. et al. 5381 (MBM)	MT, Brazil	Wetlands
	Hatschbach, G. et al. 73128B (MBM)	MS, Brazil	Sandy river soil
E. intermedia Schult.	*Brainerd, E. s.n.* (MBM80830)	U.S	Sandy river soil
E. interstincta (Vahl) Roem. & Schult.	*Pedersen, T. M. 14842* (MBM)	Argentina	Flooded low ground
	Kuniyoshi, Y. S. & Ziller, S. R. 5351 (MBM)	PR, Brazil	Hydromorphic ground, sub-forest
E. kleinii Barros	*Bona, C. & Costa, D. R. 369* (UPCB)	PR, Brazil	Temporary pond
	Bona, C. & Costa, D. R. 339 (UPCB)	PR, Brazil	Flooded land. Emergent

Species	Voucher	Origin	Habitat
*E. kurogu*wai Ohwi	*Tsuchiya, K. & Matsui, K. 3025* (MBM)	Japan	Reservoir margin
E. macrostachya Britton	*Howell, J. T. 49671* (MBM)	U.S	Field
	Seijo, G. 1308 (MBM)	Argentina	Marsh
E. maculosa (Vahl) Roem. & Schult.	*González, A. & Liesner, R. 10223* (MBM)	Venezuela	Dam margin, sandy soil
	Irwin, H. S. et al. 31024 (MBM)	BA, Brazil	River edge
E. mamillata (H. Lindb.) H. Lindb.	*Tsuchiya, K. 2390* (MBM)	Japan	Marsh
E. montana Nelmes	*Bona, C. 361* (UPCB)	PR Brazil	Dam margin. Emergent
	Bona, C. et al. 589 (UPCB)	PR, Brazil	Reservoir margin
E. montevidensis Kunth	*Pedersen, T. M. 10771* (MBM)	Argentina	Channel Margin
	Reitz, R. 808 (MBM)	SC, Brazil	Coastal zone
	Whitehouse, E. 15276 (MBM)	U.S	River edge
E. multicaulis (Sm.) Desv.	*Spichger, R. 15277* (MBM)	France	Pond margin
	Pedersen, T. M. 14188 (MBM)	Denmark	_____
E. mutata (L.) Roem. & Schult.	*Pabst, G. 7346* (MBM)	RJ, Brazil	Pond margin, sandy soil
	Reinert, B. L. & Bornschein, M. R. 65 (MBM)	PR, Brazil	Marsh
	Rigueira, D. 67938 (MBM)	BA, Brazil	Coastal zone
E. niederleinii Boeckeler	*Bona, C. 341* (UPCB)	PR, Brazil	Emerging
	Cervi, A. C. 8867 (UPCB)	PR, Brazil	_____
	Bona, C. et al. 157 (UPCB)	PR, Brazil	Pond margin
E. nudipes (Kunth) Palla	*Dombrowski, L. T. 9767* (MBM)	PR, Brazil	_____
	Delascio, F. et al. 11433 (MBM)	Venezuela	_____
E. obtusa (Willd.) Schult.	*Wofford, B. E. 80-102* (MBM)	U.S	River edge
	Morency, M. 901 (MBM)	Canada	_____
	Whitehouse, E. 16515 (MBM)	U.S	River bank, damp clay soil
E. obtusetrigona (Lindl. & Nees) Steud.	*Bona, C. et al. 368* (UPCB)	PR, Brazil	Dam margin
	Pedersen, T. M. 13389 (MBM)	Argentina	Emergent
	Bona, C. et al. 162 (UPCB)	PR, Brazil	Lagoon margin
E. ovata (Roth) Roem. & Schult.	*Fernald, M. L. 160* (MBM)	U.S	_____
	Harz, K. E.s.n. (MBM82186)	Germany	_____
	Leute, G. G. 12441a (MBM)	Australia	_____

Species	Voucher	Origin	Habitat
E. pachycarpa É. Desv.	*Pedersen, T. M. 14312* (MBM)	Chile	Wet Interdental Depressions
E. pachystyla (C.Wright) C. B. Clarke	*Davidse, G. 4297* (MBM)	Venezuela	Marsh
E. pallens S. T. Blake	*Constable, E. F. 4450* (UPCB)	Australia	River
E. palustris (L.) Roem. & Schult.	*Iltes, H. H. 606* (MBM)	U.S	Clayey soil
	Morency, M. 207 (MBM)	Canada	——
	Lampinen, R. 5099 (MBM)	Finland	Lagoon margin
E. parodii Barros	*Pedersen, T. M. 12533* (MBM)	Argentina	Wet ditch
	Pedersen, T. M. 15683 (MBM)	Uruguay	Wet soil
E. plicarhachis (Griseb.) Svenson	*Lindeman, J. C. & Haas, J. H. 868* (MBM)	PR, Brazil	——
	Lindeman, J. C. & Haas, J. H. 886 (MBM)	PR, Brazil	——
E. rabenii Boeckeler	*Andrade, P. R. P. s.n* (MBM)	PR, Brazil	——
	R. Trevisan 1165 (FLOR)	SC, Brazil	——
	Hatschbach, G. et al. 76498 (MBM)	MS, Brazil	——
	Bona, C. 263 (UPCB)	PR, Brazil	——
E. ramboana R. Trevis. & Boldrini	*Kropovickas, C. L. et al. 23800* (MBM)	Argentina	——
E. riograndensis R. Trevis. & Boldrini	*Bona, C. et al. 288* (UPCB)	PR, Brazil	——
E. rostellata (Torr.) Torr.	*Nicora, E. G. et al. 8135* (MBM)	Argentina	River bank, brackish water soils
E. sellowiana Kunth	*Stehmam, J. R. & Perdigão, G. s.n.* (MBM145828)	MG, Brazil	Emergent
	Bona, C. 370 (UPCB)	PR, Brazil	Dam margin. Amphibian
	Bona, C. et al. 156 (UPCB)	PR, Brazil	Lagoon margin
E. squamigera Svenson	*Kozera, C. & Kozera, O. P. 2660* (MBM)	PR, Brazil	——
	Cordeiro, J. et al. 1741 (MBM)	SC, Brazil	——
	Hatschbach, G. 14832 (MBM)	PR, Brazil	——
E. subarticulata (Nees) Boeckeler	*Bona, C. et al. 362* (UPCB)	PR, Brazil	Marsh, emergent
	Abreu, L. C. 344 (MBM)	SP, Brazil	Marsh
E. uniglumis (Link) Schult.	*Kukkonen, I. 10179* (MBM)	Finland	——

clades (Roalson *et al*. 2010) representing all types of aerenchyma described in Marcondes *et al.* (2021) were included. The exsiccates of the analyzed species were deposited into the FLOR, MBM, and UPCB herbaria (acronyms from Thiers, continuously updated). The seven morpho-anatomical characteristics and their variations (Tab. 2) were collected by light microscopy and scanning electron microscopy. We measured the height of the species (aerial part including stem) in about five individuals per species collected or from herbarium samples and literature data for the species not collected. The species analyzed were divided into small (< 17 cm), medium (> 17 ≤ 40 cm), and high (> 40 cm) groups (Marcondes *et al*. 2021) (Tab. 2).

Table 2 – Relationship of the characters of the stem diaphragm of the *Eleocharis* species (Cyperaceae), and their respective states. 1. Presence of secretory cells: 0 = absent; 1 = present; 2. Morphology of the diaphragm cells: 0 = lobed stellate cells; 1 = simple stellate cell; 2 = non-stellate cell; 3. Presence of microprojections on the wall of the diaphragm cells: 0 = absent; 1 = present; 4. Number of predominant layers in the diaphragm: 0 = (≤ 2); 1 = (3 to 4); 2 = (> 4); 5. Thickness of the walls of the diaphragm cells: 0 = thin; 1 = thick; 6. Morphology of the diaphragm cells of the lateral gaps: 0 = lobed stellate cells; 1 = simple stellate cells; 2 = non-stellate cells; 7. Species height: 0 = (low ≤ 16); 1 = (mean > 17 ≤ 40); 2 = (high > 40).

Species/states	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Species/states**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
E. acicularis	0	0	0	0	0	_	1	*E. macrostachya*	1	0	1	2	1	_	2
E. acuta	1	0	1	1	1	_	2	*E. maculosa*	1	1	0	0	0	_	1
E. acutangula	1	0	1	0	1	_	2	*E. mamillata*	1	0	1	1	1	_	2
E. albida	1	0	1	1	0	_	1	*E. montana*	1	0,1	0	2	1	0,1	2
E. bonariensis	1	0	1	0,1	0	_	2	*E. montevidensis*	1	0	1	2	1	_	1
E. compressa	1	0	1	0	0	_	0	*E. multicaulis*	1	0	1	1	1	_	1
E. congesta	0	0	1	1	1	_	1	*E. mutata*	1	0	1	2	1	_	2
E. contracta	1	0	1	2	1	?	2	*E. niederleinii*	1	2	1	0	0	_	1
E. cylindrica	0,1	0,1	0	1	1	_	0	*E. nudipes*	1	0	1	1	1	_	2
E. cylindrostachys	0	0	1	2	1	_	2	*E. obtusa*	1	0	1	2	1	_	0
E. densicaespitosa	1	0,1	1	0,1	0,1	_	1	*E. obtusetrigona*	0,1	0	1	0	1	_	2
E. dombeyana	1	0	1	1	1	_	0	*E. ovata*	1	0	1	1	1	_	2
E. dunensis	0,1	2	?	0	1	_	1	*E. pachycarpa*	1	0	1	1	0	_	1
E. elegans	1	0,1	1	2	1	1,2	2	*E. pachystyla*	1	0	1	1	0	_	1
E. endounifascis	1	0,1	1	2	1	0,1	2	*E. pallens*	0,1	0	1	1	1	_	0
E. engelmannii	1	0	0	1	1	_	1	*E. palustris*	1	0	1	2	1	_	2
E. equisetoides	1	0	1	2	1	?	2	*E. parodii*	1	0	1	1	0	_	2
E. erythropoda	0,1	1	1	1	1	_	1	*E. plicarhachis*	0,1	0	0	1	1	_	2
E. exigua	0	0,2	0	0	0	_	0	*E. rabenii*	1	2	0	0	0	_	1
E. filiculmis	1	1	1	1	1	_	0	*E. ramboana*	1	0,2	1	0	0	_	0
E. flavescens	1	0	1	1	1	_	0	*E. riograndensis*	1	2	0	0,1	0	_	1
E. geniculata	1	0,1	1	1	1	_	1	*E. rostellata*	1	0	1	1	1	_	0
E. hatschbachii	1	1	0	0	0	_	?	*E. sellowiana*	0,1	0,1	1	0	1	_	2
E. intermedia	1	0	1	1	1	_	0	*E. squamigera*	0,1	0	1	1	1	_	2
E. interstincta	0,1	0	1	1	1	0	2	*E. subarticulata*	1	0	1	1	0	_	1
E. kleinii	1	0	0	?	?	_	2	*E. uniglumis*	1	0	1	0	0	_	0
E. kuroguwai	0	1	0	2	1	1	2								

(?) Feature that could not be identified.

Microscopic analysis

Anatomical analyses of the aerenchyma were performed with material fixed in FAA70 (Johansen 1940) and exsiccated material rehydrated with 30% ammonium hydroxide for three hours (Toscano de Brito 1996). After fixation and hydration, both materials were stored in 70% ethanol. For all species, transverse and longitudinal sections were made in the mid and basal regions of the organ. Permanent slides were prepared from material processed and embedded in Historesin Leica®, following the manufacturer's guidelines. The blocks were sectioned using a 7-µm-thick rotating microtome, and the blades were stained with Toluidine Blue (O'Brien *et al*. 1964). Ferric chloride 10% (Johansen 1940) was used to identify phenolic compounds, and we used Lugol (Johansen 1940) to detect starch grains. The analyses were performed under an Olympus microscope (BX41TF) with an attached camera (Olympus SC30) for image capture. Measurements were made under a microscope with an ocular fitted with a calibrated scale via a Zeiss 5 + 100/100 micrometric blade.

For scanning electron microscopy (SEM) analysis, the fixed or hydrated samples, as described above, were dehydrated in an ethylic series and subjected to the CO_2 critical point method (BAL-TEC CPD030 Critical Point Dryer), adhered in metallic support with adhesive copper tape and metalized with gold (BALZERS SCD030). The analyses were done using the scanning electron microscope (JEOL JSM-6360LV Scanning Electron Microscope) at the Centro de Microscopia Eletrônica of UFPR (CME).

Phylogenetic analysis

The reconstruction of the ancestral states by parsimony was analyzed to evaluate the evolution of the characteristics resulting from the anatomical diagnosis. We employed the strict consensus tree used by Marcondes *et al*. (2021) in the analysis of *Eleocharis* stem aerenchyma patterns; this tree was generated in the TNT (Tree analysis using New Technology) with ITS1, 5.8S, and ITS2 nuclear DNA sequences and trnC-ycf6 and ycf6-psbM from the chloroplast DNA. The reconstruction was done by parsimony in the program Mesquite v. 3.03 (Maddison & Maddison 2015). The characteristics were coded discretely, and their character states are presented in Table 2.

Results

In *Eleocharis* stems, gaps of different dimensions are transversely divided by diaphragms with one or more layers of cells (Fig. 1a-d). In spongy-pattern aerenchyma, these layers are arranged at different heights in the stem (Fig. 1a-b); in septate-pattern aerenchyma, the layers divide the stem into segments with wide central diaphragms (Fig. 1c). The cells that make up the diaphragms are generally stellate (Fig. 1c,e-f) with different morphologies according to the species. The diaphragms differ in the number of cell layers, size and shape of the intercellular spaces, cell morphology, wall thickness, microprojection presence, and presence of secretory cells with phenolic compounds (Tab. 2). The presence of starch (Fig. 1g) was the only characteristic that diverged between the mid and basal regions of the stem as it was present in the base but absent in the mid-region.

The number of cell layers in the diaphragm varies between species and can be thin, with up to two layers (Fig. 1h); medium, with three to four layers (Fig. 1g, i); or thick, with more than four layers (Fig. 1j-k). Although the thickness of diaphragm layers was slightly variable in most species, both thick and thin diaphragms occurred in some species (*Elecharis bonariensis*, *E. densicaespitosa*, and *E. riograndensis*) (Fig. 2). The thickest diaphragm was recorded in *E. endounifascis*, with approximately 30 layers. Clades 1 and 7 present species with all three states of the character clade 6 contains species with medium and thin diaphragms; and clade 4 contains only species with thin diaphragms (Fig. 2).

The cells of the diaphragm layers are superimposed, which allows for continuous intercellular spaces between the layers (Figs. 1k; 3a). The intercellular spaces may be conspicuous or not (Figs. 1e-f; 3a-f). The diaphragm cells of the analyzed species may be stellate (Figs. 1e-f; 3a) or nonstellate (Fig. 3f). Stellate cells are the most frequent morphology, and in general, these have six or eight extensions. The intercellular spaces are usually delimited by the extensions of three neighboring cells, forming a space that is approximately triangular (Figs. 1e; 3a) or rounded (Figs. 1f; 3b).

Stellate cells can be divided into two types according to the format of the extensions:

I - Lobed stellate cell. In this morphology, the end of the cell extension is dilated at the attachment site with the neighboring cell (Fig.

3a-f). The dilatation is variable in each species and may be discrete (Fig. 3a) or more pronounced (Fig. 3b). In diaphragms whose cell extensions are very dilated at their extremities, the intercellular spaces are reduced (Fig. 3c-d). This dilation can further branch out and give rise to cells with complex shapes (Fig. 3d-e). In most species, these cells have microprojections on the extension

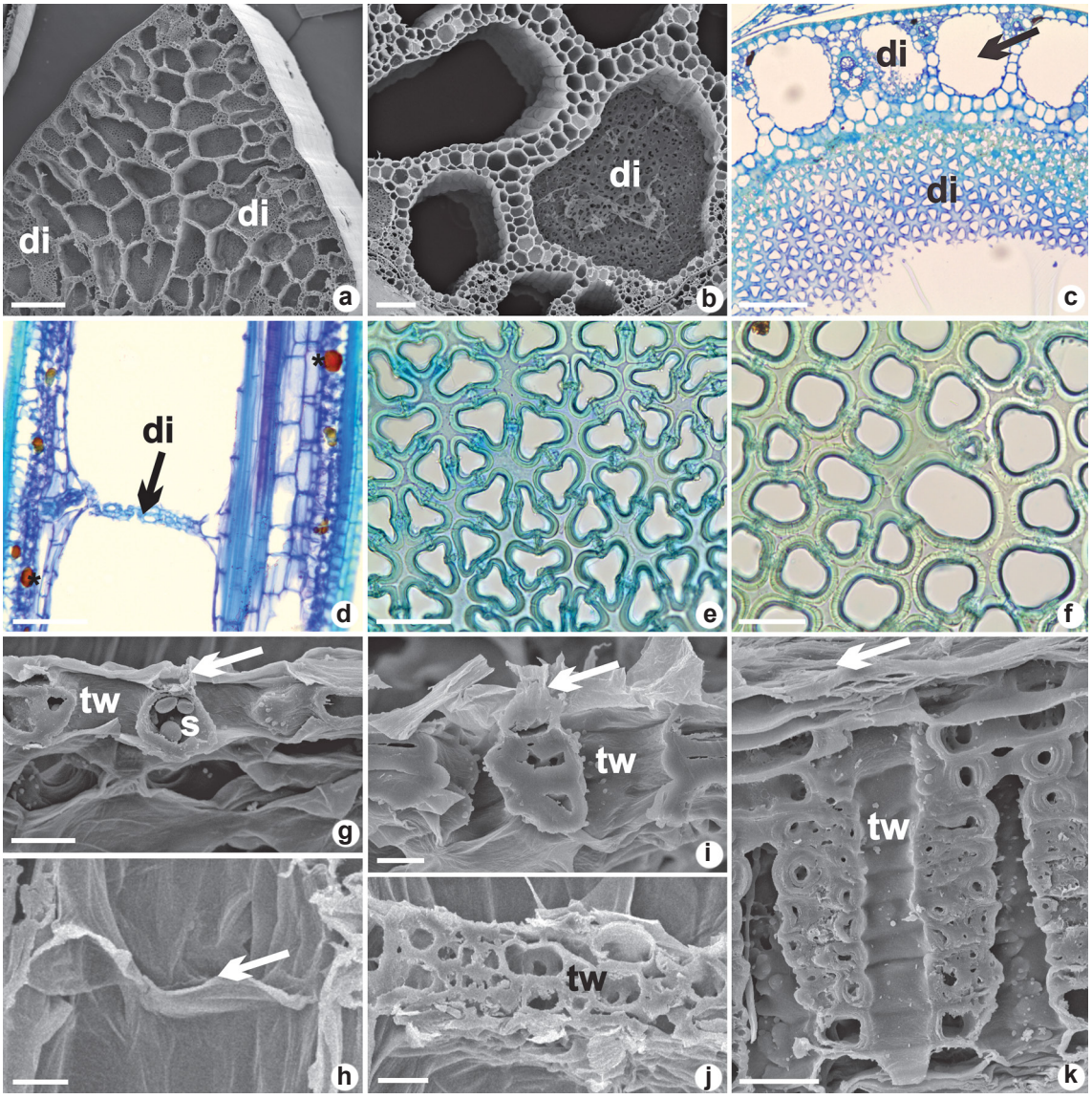

Figure 1 – a-k. Diversity in the diaphragm cells in *Eleocharis* stems; MEV (a, b, g-k), light microscopy (c-f); cross sections (a, b, c, e, f) and longitudinal sections (d, g-k) – a. general view of the spongy pattern aerenchyma, *E. acutangula*; b. gaps with diaphragm, *E. subarticulata*; c. stem with small peripheral gaps (arrow) and a large central gap with diaphragms (septate pattern), phenolic idioblasts, *E. endounifaceis*; d. a gap cross divided by the diaphragm, *E. acutangula*; e-f. diaphragms in front view with stellate cells – e. *E. interstincta*; f. *E. kuruguwai*; g. diaphragm medium with three layers of cells, the central one with thick walls and the periphery with thin walls, *E. mamillata*; h. thin diaphragm, with a layer of thin-walled cells, *E. riograndensis*; i. diaphragm medium with four layers, the two thick central, *E. palustris*; j-k. thick diaphragms, with a number of layers greater than five; j. little thick cells walls, *E. rostelata*; k. very thick cell walls, *E. equisetoides*. (Arrow = thin-walled cell; tw = cell of thick walls; s = starch; * = phenolic idioblasts; di = diaphragm). Scale bars: a = 500 µm; b, d = 100 µm; c = 200 µm, e, f = 50 µm, g = 5 µm, h, i, j = 10 µm, k = 20 µm.

walls, especially in the region of the connections among cells (Fig. 3b-d). These microprojections may or may not be linked to the microprojections of neighboring cells (Fig. 3c). Stellate cells with short and dilated extensions were recorded only in the diaphragms of *E. elegans*, *E. montana* (Fig. 3f), and *E. sellowiana*. In the other species, the extensions were well-developed, as in *E. acicularis* (Fig. 3a), *E. acutangula*, and *E. subarticulata*, among others. The other clades have both states among their species (Fig. 4), except clade 4, in which all analyzed species have microprojections in the cells.

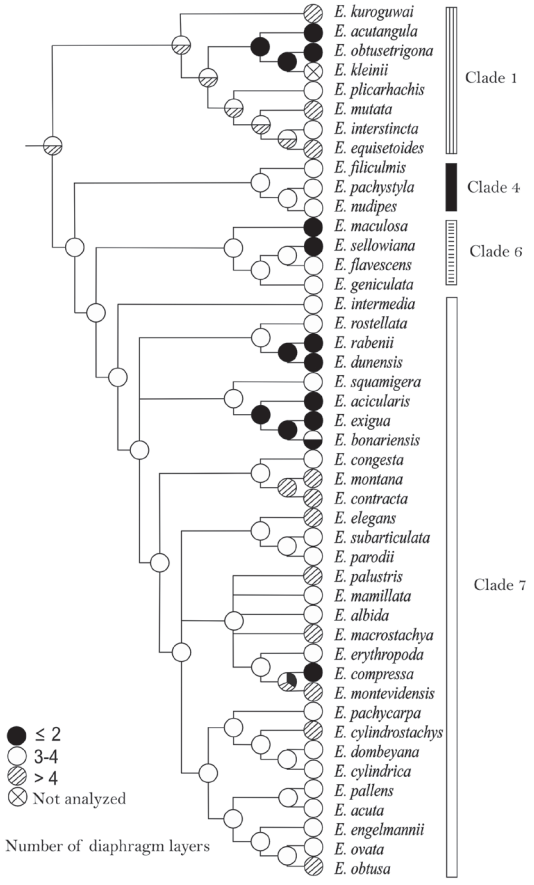

Figure 2 – Reconstruction of the ancestral state in *Eleocharis*, by the method of parsimony (Mesquite v. 3.03), based on the strict consensus tree by the TNT program (v.1.1); number of layers of the stem diaphragm, black (less than or equal to two), white (three to four), striped (greater than four) and X (not analyzed). The indicated clades were previously defined based on the phylogeny of the group (Roalson *et al.* 2010).

II - Simple stellate cell. In this morphology, the extensions of the cells do not have dilations, and in general, the microprojections are absent (Fig. 5a-b). These cells were recorded in the diaphragms of *E. cylindrica*, *E. elegans*, *E. erythropoda*, *E. filiculmis*, *E. geniculate*, *E. kuroguwai*, *E. maculosa*, *E. montana* (central lacuna), and *E. sellowiana*. In *E. kuroguwai*, the extensions of the stellate cells have little delimitation, creating circular intercellular spaces (Fig. 5b).

Nonstellate cells have irregular shapes on the transverse axis, are flattened on the longitudinal axis, and lack extensions and microprojections (Fig. 5c-d). These cells are arranged in a juxtaposed way, which results in nonobvious intercellular spaces. Nonstellate cells were found only in the diaphragms of small species (*E. exigua*, *E. dunensis*, *E. niederleinii*, *E. rabenii*, and *E. riograndensis*), and phenolic compounds may be present (Fig. 5c). Clade 7 was the only one containing all three states of the described diaphragm cell morphology, while the three other analyzed clades had species with lobed stellate cells and simple stellate cells (Fig. 6).

Diaphragm cells may have thin primary walls and lignified secondary walls of different thicknesses (Fig. 1e-k). In diaphragms with thick-walled cells, these are in the center of the layer, coated by thin-walled cells that are often torn or stretched (Figs. 1i, k; 3a-b). In diaphragms with lobed thick-walled cells, thin-walled cells may or may not exhibit microprojections. With the exception of *E. kleinii*, in which it was not possible to analyze this characteristic, the other species of clade 1 presented with thick-walled cells. The other clades presented predominantly thick-walled cells and isolated species or small groups with cells without any thickening in their diaphragms (Fig. 7). A thickened wall is the plesiomorphic state of the group, and the absence of thickening is an apomorphic state that appeared at least eight times in *Eleocharis's* history (Fig. 7).

Most of the analyzed species presented phenolic content secretory cells (as shown by a positive test for ferric chloride) distributed randomly in the diaphragm (Tab. 2; Fig. 5c). The presence of secretory cells is the plesiomorphic state of the genus, while the absence of these cells is an apomorphic state that arose at least five times. Clades 1, 6, and 7 each contained species with and without secretory cells, while species in clade 4 all have secretory cells. The nine analyzed species presented specimens with both states (Tab. 2).

Discussion

The diaphragms in *Eleocharis* are composed of one to several layers of parenchyma cells, in line with the findings of Metcalfe (1971). The ancestral state of the number of layers of the diaphragm was ambiguous; the oldest ancestor of the group could have had either medium or thick diaphragms. Fine diaphragms compose an apomorphic state in the group, which has appeared several times in the history of the genus (see Fig. 2).

The vast majority of species present lobed stellate cells. The first records of lobed stellate cells in the genus occurred in the species *E. acutangula*, *E. geniculata*, *E. intermedia*, *E. kleinii*, *E. montana*, *E. mutata*, *E. palustris*, and *E. subarticulata* (Metcalfe 1971). The morphological character of the diaphragm cells demonstrates how the ancestral state influences the lobed stellate cell. Simple stellate cells and nonstellate cells are apomorphic states, where the first appeared at least nine times, and the second appeared two times throughout the evolutionary history of the group (see Fig. 6).

Most species only have one type of morphology in their diaphragm cells, but some have different morphological types in the same individual or among specimens. This is the case for *E. montana* and *E. elegans*, which present stems with septated architectures, and the morphology of the lateral diaphragm cells (present in the peripheral gaps) differs from that of the central gap diaphragm (Marcondes *et al*. 2021).

The intercellular spaces of the species' diaphragms are triangular, as reported by Govindarajalu (1975), circular, or may not be

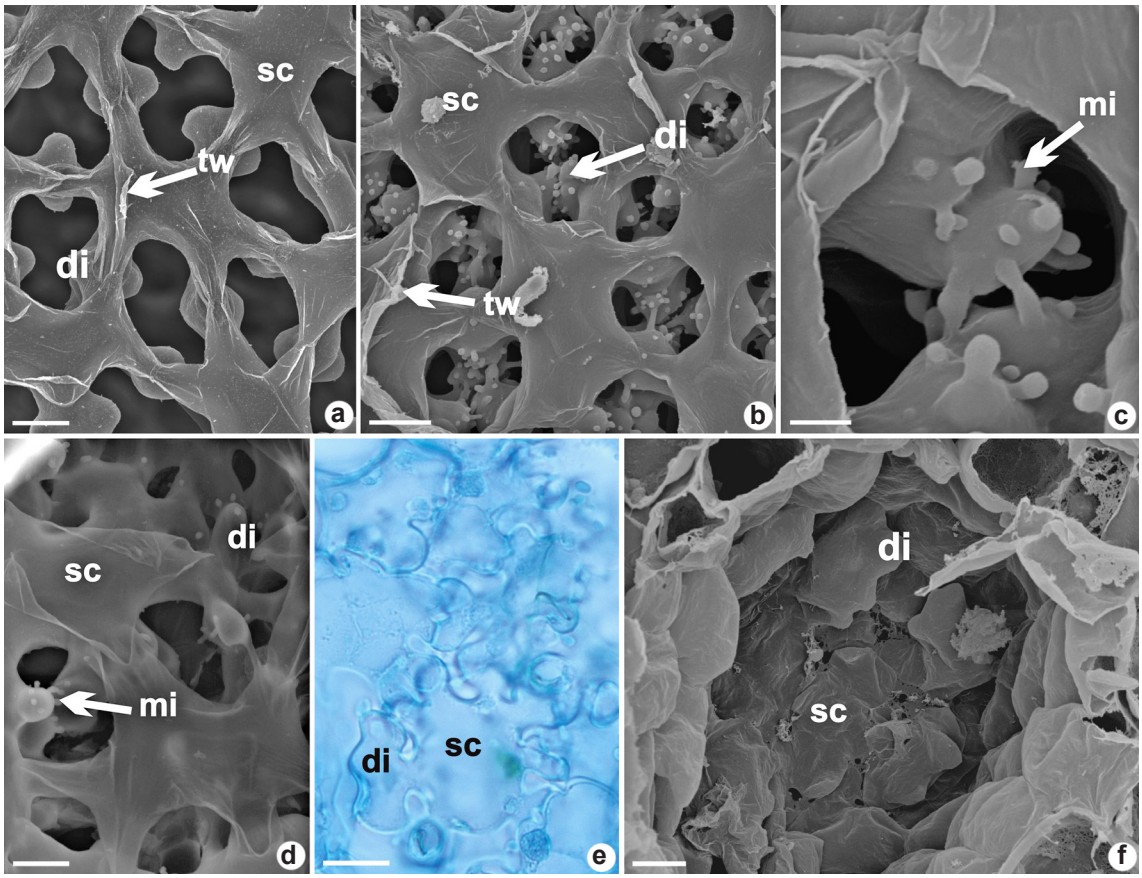

Figure 3 – a-f. Morphological variation of the stellate cells, in MEV (a-d, f) and light microscopy (e) – a. stellate cells with free intercellular space, *E. acicularis*; b. stellate cells delimiting intercellular space partially obstructed by the dilatations of the lobes, *E. subarticulata*; c. detail of connections in dilated regions *E. subarticulata*; d-e. cell with branched dilatations, *E. niederleinii*; f. detail of the short and dilated arms of the cells with arms. (sc = stellate cells; tw = thin-walled cells; di = expansion of the dilations; mi = microprojection). Scale bars: a, b, f = 10 µm; c = 2 µm; d = 5 µm; e = 20 µm.

visible. The variation of the spaces is a result of the combination of morphology, wall thickness, and cellular arrangement. Diaphragms with larger intercellular spaces are commoner in large species than in small ones. The explanation for this characteristic is given by Snow (1914), which corroborates the results of a biomechanical study by Schwendener (Schwendener *apud* Snow 1914), which relates the size of the extensions to the disparity in the speed of growth between the cells. While the stellate cells assume this morphology due to the tension they undergo during their growth, the surrounding tissue develops faster. As the growth of the diaphragm cells cannot keep pace with the external cells, they are stretched, forming the "arms" or extensions.

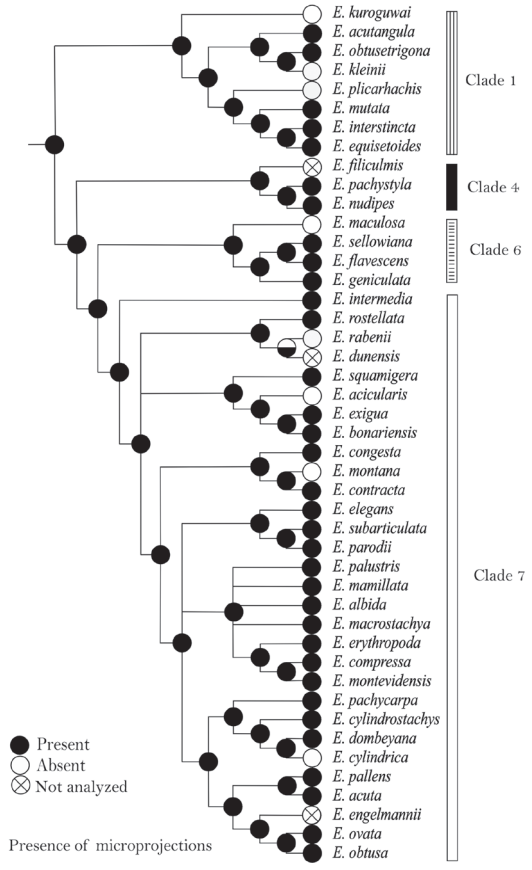

Figure 4 – Reconstruction of the ancestral state in *Eleocharis*, by the method of parsimony (Mesquite v. 3.03), based on the strict consensus tree by the TNT program (v. 1.1); presence of microprojections in the cell of stem diaphragm; black (present), white (absent), and X (not analyzed). The indicated clades were previously defined based on the phylogeny of the group (Roalson *et al.* 2010).

Diaphragms with thick-walled cells are present in most species, but diaphragms with thin-walled cells usually occur in smaller species. The medium to large species present a greater anatomical variety in the diaphragm and have a greater number of layers and greater wall thickness. This result is expected since one of the functions of the diaphragm is to provide mechanical resistance and stabilization to the organ (Snow 1914; Sculthorpe 1967; Dickison 2000; Kaul 1971, 1972). Thus, the larger stems have more developed diaphragms and thicker walls.

The morphological variations found in the shape of the diaphragm cells in *Eleocharis* were similarly described in other species (Snow 1914; Govindarajalu 1975; Bona & Alquini 1995a, b). This result demonstrates that diaphragms, although having characteristics common to species, such as the presence of stellate cells, may have individual peculiarities or peculiarities in groups of species (*i.e.*, the branching of the extensions). The distinctive shape found in *E. kuroguwai* (Fig. 5b), which gives rise to circular intercellular spaces, is an example of a morphology that was recorded in this species and *E. sphacelata* R. Br. (Sorrell *et al*. 1997). Diaphragms without stellate cells, as reported here and by Metcalfe (1971), also delimit a small group of species among those analyzed (*E. exigua*, *E. dunensis*, *E. niederleinii*, *E. rabenii*, and *E. riograndensis*), all of which are medium-sized and small (Tab. 2). Unlike the diaphragms of some species that present vascular bundles (Kaul 1971), the analyzed species of *Eleocharis* all lack these bundles.

The presence and function of microprojections in diaphragm cells have not yet been investigated in academic work. Studies of their ontogenesis are needed to understand their development and function. We can affirm that these structures are associated with lobed stellate cells in *Eleocharis*. They are often united with the microprojections of neighboring cells, a characteristic that is present in most species, genetically well-fixed, and can be used for taxonomic purposes. Microprojections in stellate cells have been present throughout the evolutionary history of the genus since its earliest ancestor. Our results demonstrate that the absence of microprojections appeared independently at least eight times in the group's history (see Fig. 4).

Diaphragms may contain chloroplasts, laticifers, and starch (Snow 1914). Secretory cells in the stem are mentioned in several species of *Eleocharis* (Metcalfe 1971), may also appear in the stolon and rhizome (Hess 1953), and, according to Govindarajalu (1975), may indicate the presence of tannins. In *Eleocharis*, the constant presence of phenolic compounds is not of taxonomic importance but is a function of herbivory prevention (Coley 1988) and resistance to infection by microorganisms such as fungi and bacteria (Levit 1971), which are common in these species' aquatic environments.

No unique anatomical characteristics were found among the members of the same clade. Some identified characteristics were frequently present within a genus, while others appeared more discretely. The presence of microprojections in the diaphragm cells and the thickening of their walls are the most relevant characteristics to aid in a genus's classification, as they do not show variations within a species. Both can delimit small groups in the different clades, such as those cited in the results and exemplified in the characteristic reconstruction trees.

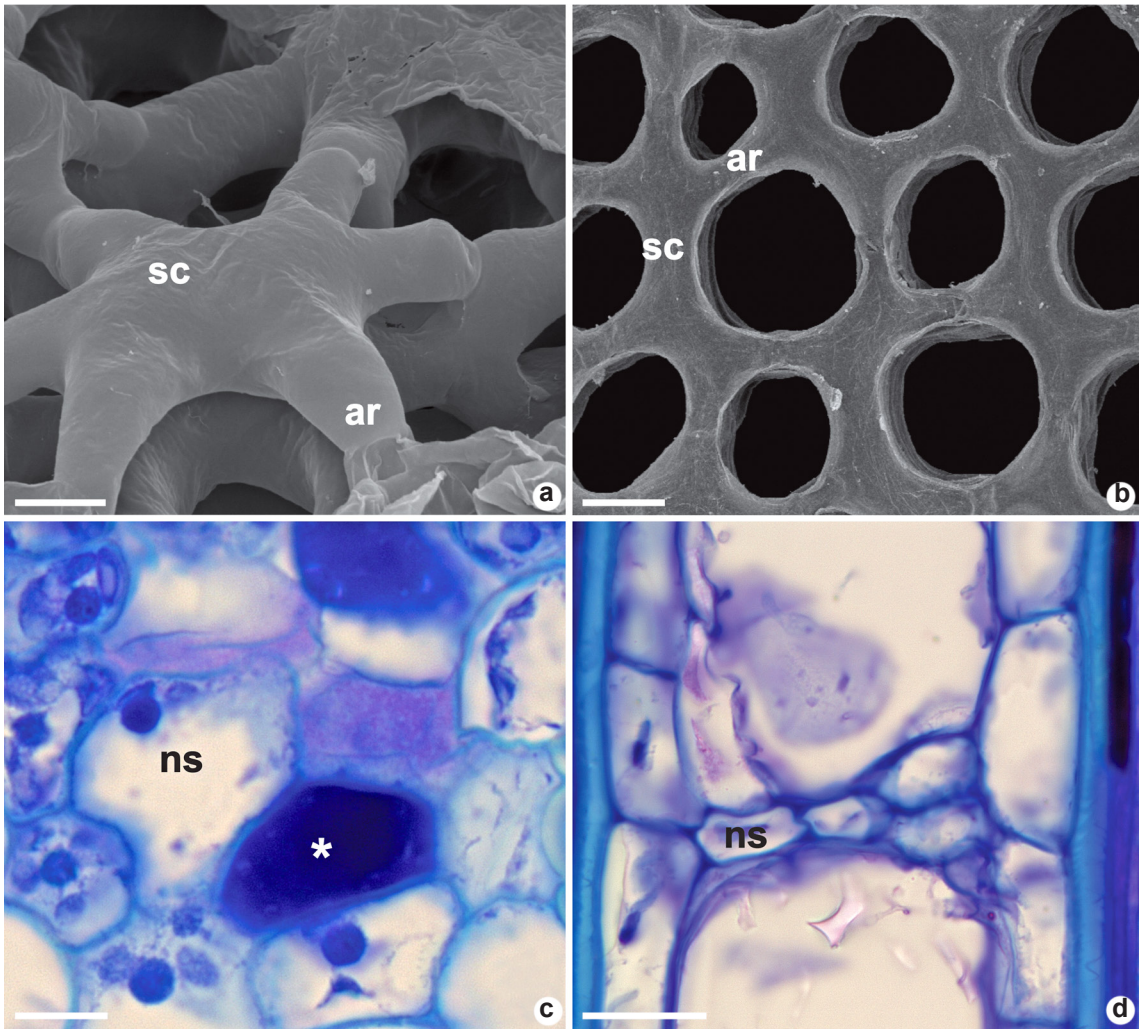

Figure 5 – a-d. Diaphragm cell morphology in *Eleocharis* stems; in MEV (a, b) and light microscopy (c, d) – a-b. stellate cells with simple extensions – a. straight extensions, *E. montana*; b. cell with body and a little distinct extensions, *E. kuroguwai*; c-d. non-stellate cell – c. diaphragm in frontal view showing the shape of the cells slightly irregular and juxtaposed, with phenolic idioblasts (*), *E. rabenii*; d. longitudinal aspect of the diaphragm, *E. dunensis*. (sc = stellate cell; ns = non-stellate cell; ex = extensions of the cells). Scale bars: a = 5 µm; b = 25 µm; c = 10 µm; d = 20 µm.

The evolutionary history of the diaphragm in *Eleocharis* reveals the genus's anatomical variations; however, the plesiomorphic states of the analyzed characteristics are still the most common among its species. The typical *Eleocharis* diaphragm comprises thick-walled, lobed stellate cells with microprojections, secretory cells, and more than three layers of cells. None of the species studied presented all the characteristics' apomorphic states. In the evolution of all the analyzed characteristics, the apomorphic state is the simplest anatomy, which demonstrates that the structure of the diaphragm is simplifying, which favors the species by reducing their energy expenditure in forming diaphragms.

We can conclude that although the species of the genus live in an environment of high plasticity, they developed few variations in the anatomical and micromorphological structures of their diaphragms. None of the characteristics raised are exclusive among the clades, but they contribute to the characterization of the groups and can collaboratively allow for the understanding of phylogenetic relationships in future studies of the genus. The evolution of the raised characteristics demonstrates that the *Eleocharis* diaphragm is becoming simpler, but the plesiomorphic characteristics are still the most frequent within the group.

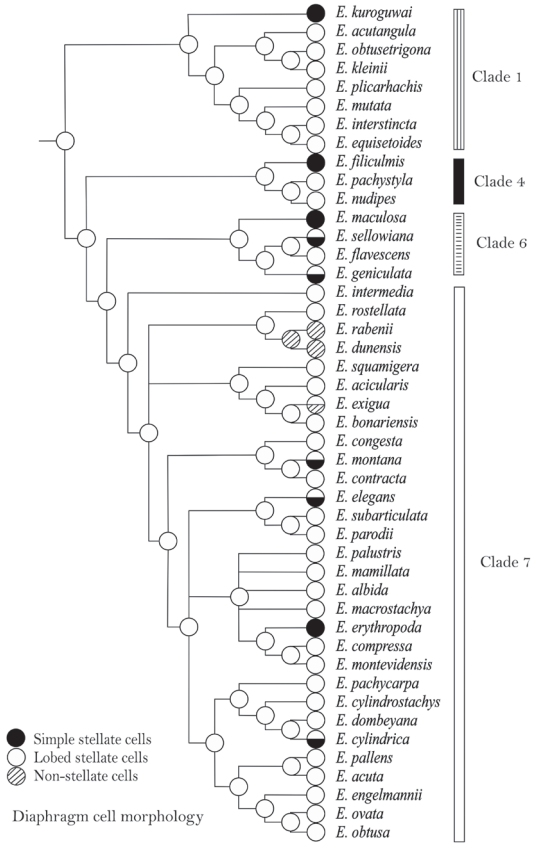

Figure 6 – Reconstruction of the ancestral state in *Eleocharis*, by the parsimony method (Mesquite v. 3.03), based on the strict consensus tree by the TNT program (v.1.1); Morphology of the stem diaphragm cell; black (simple stellate cell), white (lobed stellate cell), and striped (non-stellate cell). The indicated clades were previously defined based on the phylogeny of the group (Roalson *et al.* 2010).

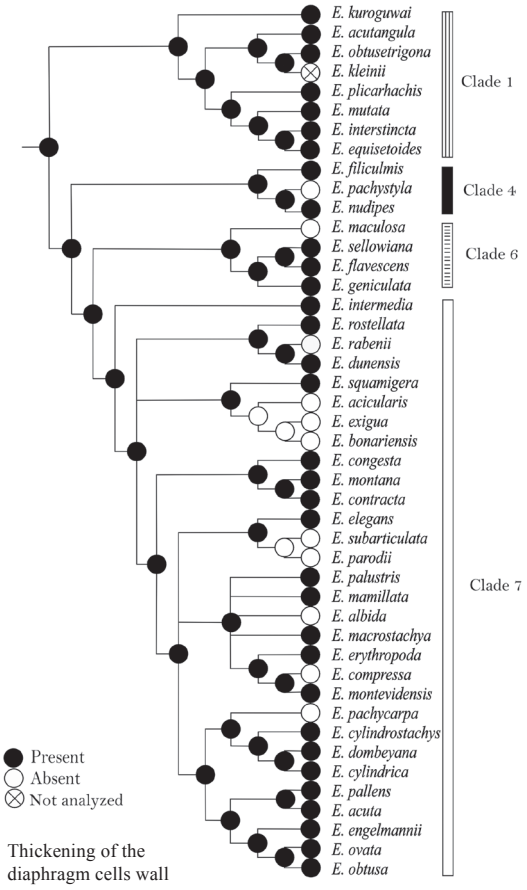

Figure 7 – Reconstruction of the ancestral state in *Eleocharis*, by the parsimony method (Mesquite v. 3.03), based on the strict consensus tree by the TNT program (v.1.1); Thickening of the walls of the stem diaphragm cells; black (present), white (absent) and X (not analyzed). The indicated clades were previously defined based on the phylogeny of the group (Roalson *et al.* 2010).

Acknowledgements

This study was financed in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 - MSc scholarship to RMP; and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We thank Dr. Eric H. Roalson (School of Biological Sciences, Washington State University), for making the data for the phylogeny available; to MBM Herbarium employees, for permission to collect samples; and the Center of Electron Microscopy of UFPR, for the use of laboratories and equipment.

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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Area Editor: Dr. João Paulo Basso-Alves
Received on May 30, 2023. Accepted on November 16, 2023.