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Ferns and Lycophytes as new challenges

Diversity of tracheary elements of Aspleniaceae

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Abstract
Tracheary elements can be classified as tracheids or vessel elements. In ferns, both cell types and even cells with intermediate characteristics can be observed. For a long time, the methods employed in the study of these cells were questioned and consequently their classification as well. In the present work, we analyze the stem tracheal elements of 13 Aspleniaceae species, occurring in different habitats and belonging to different clades. The samples were cut, dried on a hotplate, metallized, and analyzed in SEM. In general, the *Asplenium* species analyzed contain elongated conducting cells with oblique end walls, with scalariform thickening on all faces of the cell. The presence of pit membranes, however, varies greatly among species, it being possible to observe species with tracheids, with intermittent perforation plates, and with vessel elements.
Key words: ferns, pit membrane, SEM, vessel elements, tracheids.

Resumo
Os elementos traqueais podem ser classificados como traqueídeos ou elementos de vaso. Nas samambaias podem ser observados ambos os tipos de células e até mesmo células com características intermediárias. Durante muito tempo os métodos empregados no estudo dessas células foram questionados e consequentemente a sua classificação também. No presente trabalho analisamos os elementos traqueais do caule de 13 espécies de Aspleniaceae, ocorrendo em diferentes habitats e pertencentes a diferentes clados. As amostras foram cortadas, secas em placa quente, metalizadas e analisadas em MEV. Em geral, as espécies de Asplenium analisadas contêm células condutoras alongadas com paredes terminais oblíquas, com espessamento escalariforme em todas as faces da célula. A presença de membranas de pontoações, entretanto, varia muito entre as espécies, sendo possível observar espécies com traqueídeos, com placas de perfuração intermitentes e com elementos de vasos.
Palavras-chave: samambaias, membrana de pontoação, MEV, elementos de vaso, traqueídeos.

Introduction

The term "tracheary element" is derived from "tracheae", a name originally applied to certain elements of the primary xylem that resemble insect trachea (Esau 1965). Tracheary elements are more or less elongated cells, with lignified walls, which die at maturity. Cell differentiation appears to occur concurrently with the programmed cell death (PCD), in which the tonoplast ruptures and cellular components are rapidly degraded (Escamez & Tuominem 2014).

Two fundamental types of tracheary elements occur in the xylem, tracheids and vessel elements. They differ from each other because tracheids are imperforate cells that contain only pit pairs (regions without a secondary wall) in their common walls, while vessel elements still have perforations, areas without pit membrane (primary wall and middle lamella) (Evert 2006).

As the pit membrane is a very important feature for defining the type of tracheary element, there has been intense discussion this topic. Some studies have discussed whether the techniques employed in the analysis of the pit membranes were reliable; whether they could have removed portions of the membrane or even left it porous in appearance (Carlquist & Schneider 2007; Jansen *et al.* 2009; Hong-Fang & Ren 2011). It is now known

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that the different techniques used in SEM little alter the pit membrane, making this type of microscopy the most suitable for the study of tracheal elements.

The tracheary elements of ferns have been well investigated, mainly by Carlquist & Schneider (1998 a,b 1999a,b, 2000a,b,c,d, 2001, 2007), who intensively used SEM and documented them in different families and genres of ferns: *Woodsia*, Osmundaceae, Schizaeaceae, Marattiaceae, *Ceratopteris*, Blechnaceae, Cyatheaceae, Dicksoniaceae, Pteridaceae, Hymenophyllaceae (Carlquist & Schneider 1998a,b, 1999a,b, 2000a,b,c; Carlquist *et al*. 2000), *Microgramma*, *Dicranopteris*, Aspleniaceae, Elaphoglossaceae and Vittariaceae (Schneider & Carlquist 1998a,b, 1999). More recently, Laskar *et al.* (2020) studied the vessel elements of thelypteroid ferns.

Ferns may present tracheids, vessel elements, or even both cell types in a single plant. The distinction between these cell types, however, may not be so clear. Some cells present intermediate characteristics that make it difficult to classify them. In some cases, it is possible to observe incipient perforation plates in which the end wall membranes have undergone intense lysis but not been completely removed. Thus, it is not always easy to name the conduction cells in the group because in many cases it is not possible to classify them as tracheids or vessel elements (Carlquist & Schneider 2001). In addition, the tracheary elements of ferns may have vessel elements with distinctive perforation plates, multiple end-wall perforation plates, pit dimorphism, lateral-wall perforation plates, intermittent perforation plates, vessel elements and tracheids, porose pit membranes, and incipient perforation plates.

The present work aims to describe the tracheary elements of 13 species of Aspleniaceae.

Material and Methods

Thirteen species from different habitats and occurring in different environments were selected (Tab. 1). Most species occur in a humid environment, and the collected sample of *Hymenasplenium triquetrum* occurred alongside a waterfall. The sample of *Asplenium douglasii* occurred in a rocky outcrop. Fresh aerial stem and rhizome of five specimens were collected for each species. The samples were cut in the nodal and intermodal region.

Carlquist & Schneider (2007) used three different methods to process their samples: drying of sections with critical point, maceration, and hand-cut sections dried on a hot plate. The last method, according to the authors, presented better results with a much fewer artifacts and was, therefore, the method chosen for this study.

Longitudinal sections about 1 mm thick were hand-made using razor blades. These sections underwent three changes of distilled water to remove water-soluble substances, starch grains, and surface contaminants. Sections were then placed between pairs of cleaned blades with light pressure made by clips and then dried on a 60 °C hot plate (Carlquist & Schneider 2007). Next, the fragments were affixed with carbon tape on their own supports and covered with a 20 nm layer of gold (Bal-Tec Sputer Coater SCD 050). After that the samples were observed and electromicrographed under JEOL 5310 and JEOL JSM 639 OLV scanning electron microscopes at an acceleration voltage of 25 KV.

Results

In general, the stems of the *Asplenium* species analyzed contain elongated conducting cells with oblique end walls; the number of lateral walls, however, can range from 3 to 5. Scalariform thickening is observed on all faces of the cell. The presence of pit membranes, however, and hence the cell type, varies greatly between species (Tab. 1).

Tracheids

In most species, the tracheary elements observed in the xylem of the stem present a whole pit membrane on all the faces of the cell in *Asplenium auritum* (Fig. 1a), *A. harpeodes*, *A. inaequilaterale*, and *A. mourai* (Fig. 1b), and in *A. douglasii* (Fig. 1c) at the end walls of the cells, characterizing the tracheids. In *A. pseudonitidum* and *A. regulare*, the pit membrane was more porous in some parts (Fig. 1d).

Intermittent perforation plates

Asplenium scandicinium (Fig. 2a), *A. raddianum* (Fig. 2b), and *A. unisseriale* (Fig. 2c) show tracheary elements of the stem with lateral walls containing porous pit membranes with pores of varying sizes. Pit membrane remnants tend to be strands oriented in an axial direction. At times, there is very little of the pit membrane and intermittent perforations are evident. In *A. raddianum* and *A. unisseriale*, we observed spots with and without pit membranes alternating on the same face of the wall (Fig. 2b-c).

Table 1 – Species of *Aspleniaceae*, with respective numbers of record, location, habitat and environment, type of cell, perforation plate and pit membrane.

Species	**Voucher**	**Habitat**	**Municipality**	**Type**	**Pit membrane**	**Perforation plate**
Asplenium auritum Sw.	*Damasceno 425* (RB)	rupicolous	Itatiaia	tracheids	nonporose	-
Asplenium douglassi Hook. & Grev.	*Bovini 3689* (RB)	terrestrial	Ilhas Cagarras	tracheids	nonporose	-
Asplenium harpeodes Kunze	*Damasceno 352* (RB)	rupicolous	Itatiaia	tracheids	porous	-
Asplenium inaequilaterale Willd	*Sylvestre 2209* (RB)	rupicolous	Cachoeiras de Macacu	tracheids	porous	-
Asplenium monanthes L.	*Sylvestre 2189* (RB)	rupicolous	Itamonte	vessel elements	porous	Multiple end-wall
Asplenium mourai Hieron.	*Damasceno 402* (RB)	rupicolous	Itatiaia	tracheids	nonporose	-
Asplenium pseudonitidum Hook.	*Sylvestre 2175* (RB)	terrestrial	Teresópolis	tracheids	porous	-
Asplenium raddianum Gaudich.	*Sylvestre 2179* (RB)	epiphyte	Itatiaia	tracheids	porous	Intermitent
Asplenium regulare Sw.	*Jascone 1179* (RB)	rupicolous	Guapimirim	traqueids	porous	-
Asplenium scandicinum Kaulf.	*Damasceno 392*	epiphyte	Itatiaia	tracheids	porous	Intermitent
Asplenium serratum L.	*Pellegrini 285* (RFA)	rupicolous	Nova Iguaçu	vessel elements	porous	Multiple end-wall
Asplenium unisseriale Raddi	*Sylvestre 2174*	terrestrial	Teresópolis	tracheis	porous	Intermitent
Hymenasplenium triquetrum (N. Murak. *et* R.C. Moran) L. Regalado & Prada	*Sylvestre 2208* (RB)	rupicolous	Cachoeiras de Macacu	vessel elements	porous	Multiple end-wall

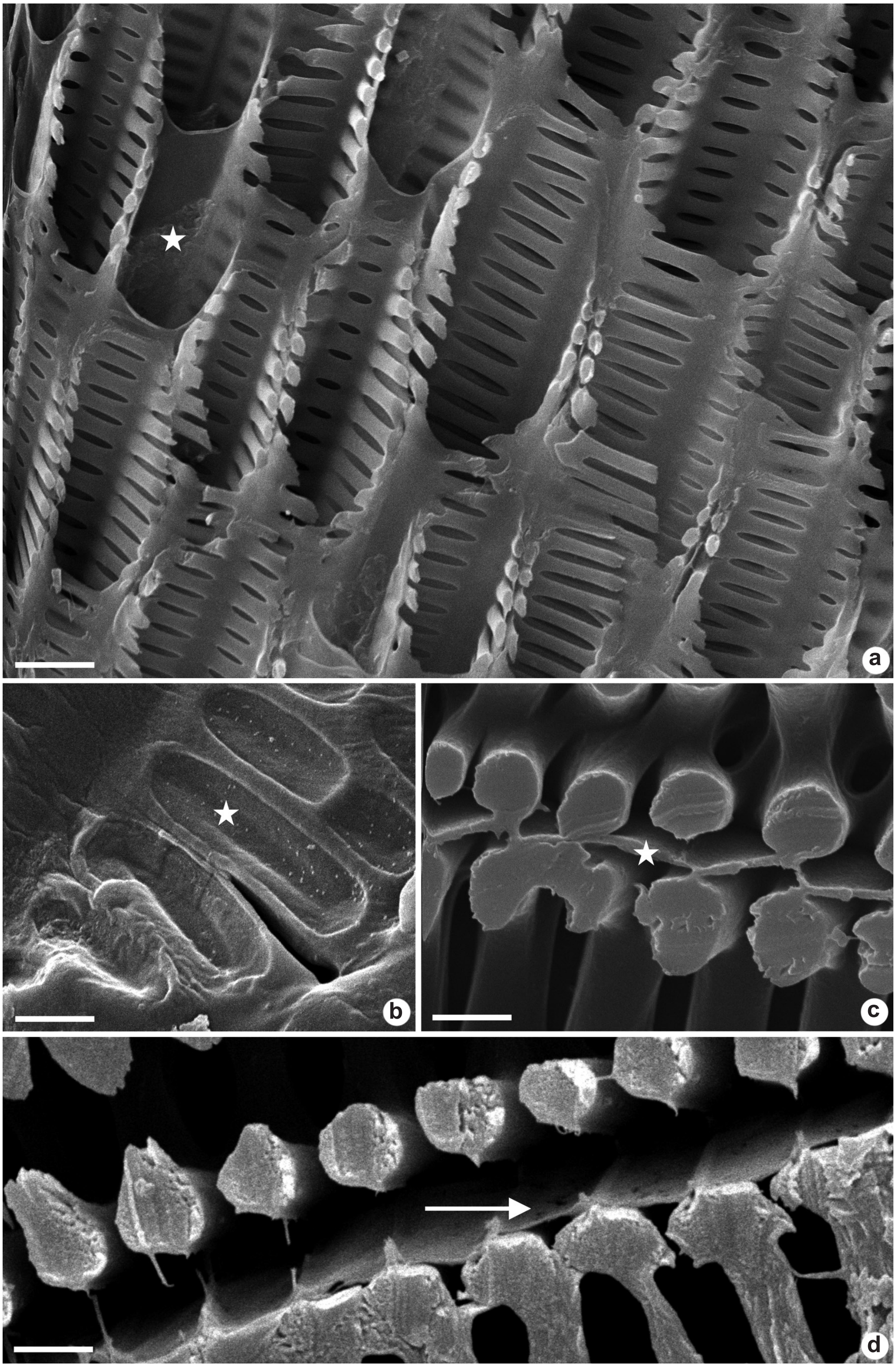

Figure 1 – a-d. Traqueary elements of Aspleniaceae species with pit membrane – a. *Asplenium auritum*; b. *A. mourai*; c. *A. douglasii*; d. *A. regulare*. (Arrows = membrane; star = porous pit membrane). Scale bars: a = 20 µm; b = 5µm; c = 2µm; d = 2µm.

Vessel elements (multiple end-wall perforation plates)

The vessel elements has multiple end-wall peforation plates (Fig. 3). *Asplenium monanthes* (Fig. 3d) shows tracheary elements very similar to those observed in *Hymenasplenium triquetrum* (Fig. 3a, c) with a terminal portion of three oblique faces, in which the pit membranes seem to be totally absent. In *A. serratum* (Fig. 3b), the terminal walls of the conducting cells also lack a pit membrane and, on rare occasions, a remnant can be observed in the side walls.

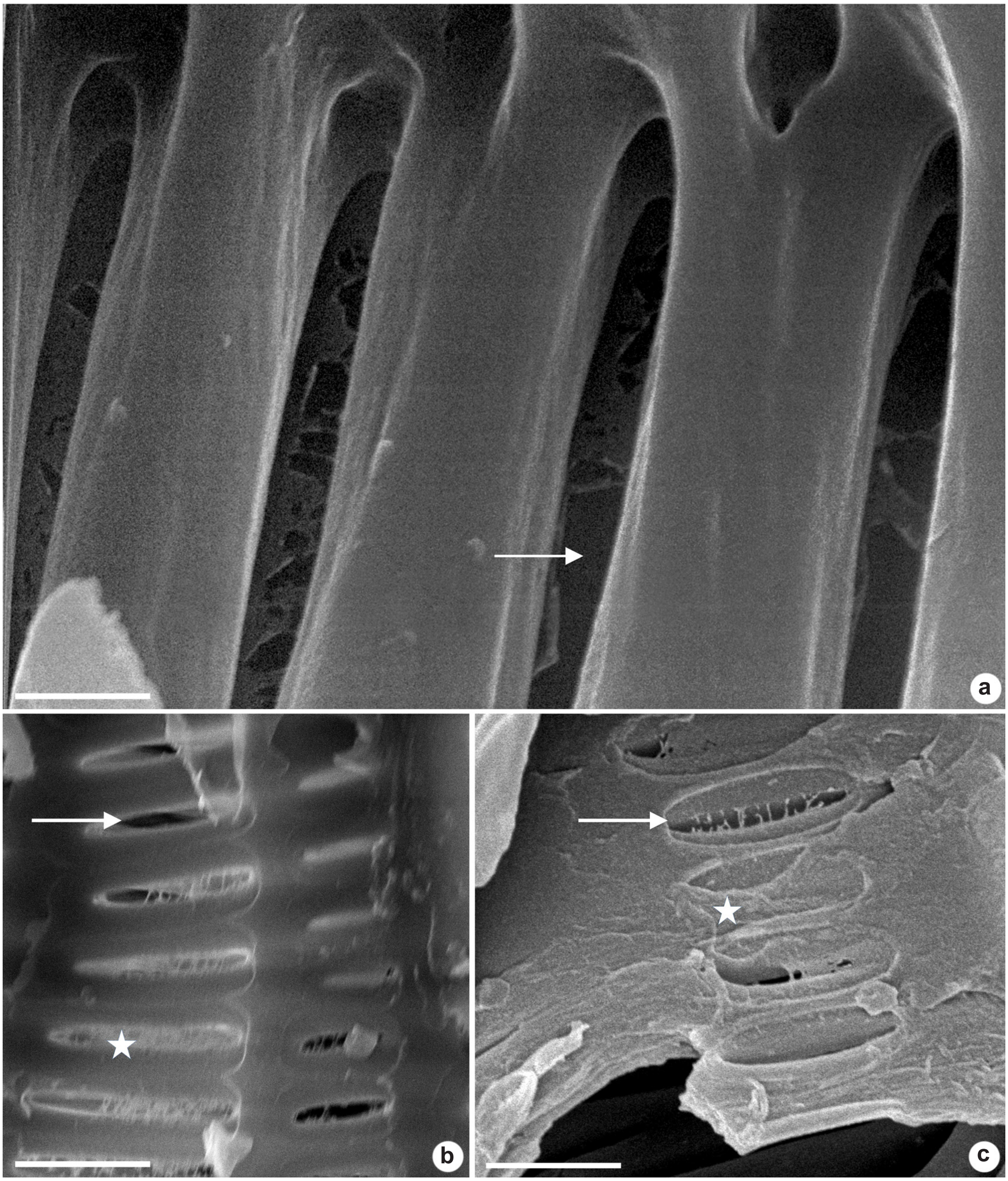

Figure 2 – a-c. Traqueary elements of Aspleniaceae species – a. *Asplenium scandicinium*; b. *A. raddianum*; c. *A. unisseriale*. (Arrows = holes; star = porous pit membrane). Scale bars: a = 2µm; b-c = 5µm.

Discussion

Pit membranes: we can trust our results

The presence or absence of the pit membrane is often a controversial subject. This stems from the fact that the methods employed in its analysis can be aggressive, leading to its removal.

In 2007, Carlquist & Schneider decided to review the methodology used in their series of studies on fern tracheal elements. The authors concluded that, due to the maceration techniques that had been used up to then, their observations of multiple perforation plates, lateral perforation plates, and intermittent perforation plates should be considered erroneous. They, however, offered a less aggressive alternative technique suggestion.

In 2009, Jansen *et al.* decided to undertake an extensive study on the variation of the pit membranes in angiosperms using MEV and MET. The researchers concluded that many of the large pores observed with SEM are the result of layers being removed from the membranes. However, in many cases, pores have been observed in apparently intact pit membranes. In some cases, large pores have been observed in pit membranes seen through the inner aperture of the pit channel of an undamaged secondary wall. These observations indicate that large diameter pores were not always caused by the removal of material from the wall.

Finally, in 2011, Hong-Fang & Ren demonstrated that treatments before SEM, which may include air-drying, fixing, dehydrating, xylene infiltrating, paraffin wax embedding, sectioning, dewaxing, and treating with Jeffrey's Fluid, have little effect on proving the absence of pit membranes or demonstrating membrane remnants. Thus, the authors concluded that the non-observation of the membrane should be treated as real evidence of its absence.

In the present work, we observed nonporous membranes, porous membranes, large-hole membranes forming intermittent perforation plates, and membrane-free areas characterizing perforation plates. We believe that the observed results are reliable for the following reasons: 1- the 13 species analyzed received the same treatment, and it is unlikely the membrane would remain intact in certain species and not in others due to the methodology; 2- we used the same less aggressive method successfully employed by Carlquist & Schneider (2007); 3- Hong-Fang & Ren (2011) demonstrated that the methods used in SEM do not compromise the membrane, and its absence and even the presence of porous membranes should be interpreted as such.

Perforation plates and intermittent perforation plates

In *Asplenium monanthes* and *Hymenasplenium triquetrum* it was not possible to observe the pit membrane, even after choosing new samples and repeating the whole process. In turn, in *A. serratum*, in some regions, remnants of the membrane could be seen. In these species, it is clear that intense lysis occurred to perforation plates.

In other species (*Asplenium scandicinium*, *A. raddianum*, and *A. unisseriale*) it is possible to observe large pores in the pit membranes, which leaves room for doubt. According to Carlquist & Schneider (2007), by using the term intermittent perforation plate, a transition is recognized even if no distinction is made between tracheids and vessel elements in these transitional instances.

On the diversity of cell types in Aspleniaceae

There is a diversity of tracheal elements in Aspleniaceae, as it is possible to observe tracheids, elements with intermediate characteristics (intermittent perforation plates) and vessel elements. Some questions arise from this observation. 1- Is it possible that the type of tracheal element correlates with the plant habitat? 2- Is it possible that the vessel elements appear only once in the family? 3- Can the arrangement of tracheal elements and parenchyma cells in the xylem interfere with the tracheal element type?

As for the first question, it is widely known that epiphyte plants usually have characteristics that allow fast conduction of water (*e.g.*, the presence of large perforation plates). To investigate such a possibility in ferns, Carlquist & Schneider (1999b) analyzed some species of epiphyte plants, including *Asplenium nidus* Sw. Some tracheary elements end walls have pit membranes with discrete porosity; many have more porous membranes. Some areas may present incipient pores that may be the beginning of the formation of distinct perforation plates in the *A. nidus* rhizome. The authors, however, did not observe any modifications or characteristics that could be correlated with conductivity rates. Luna *et al.* (2010) studied five species of Asplenium and concluded that the presence of porose pit membranes in tracheids was not related to habitat or habit type (epiphytic,

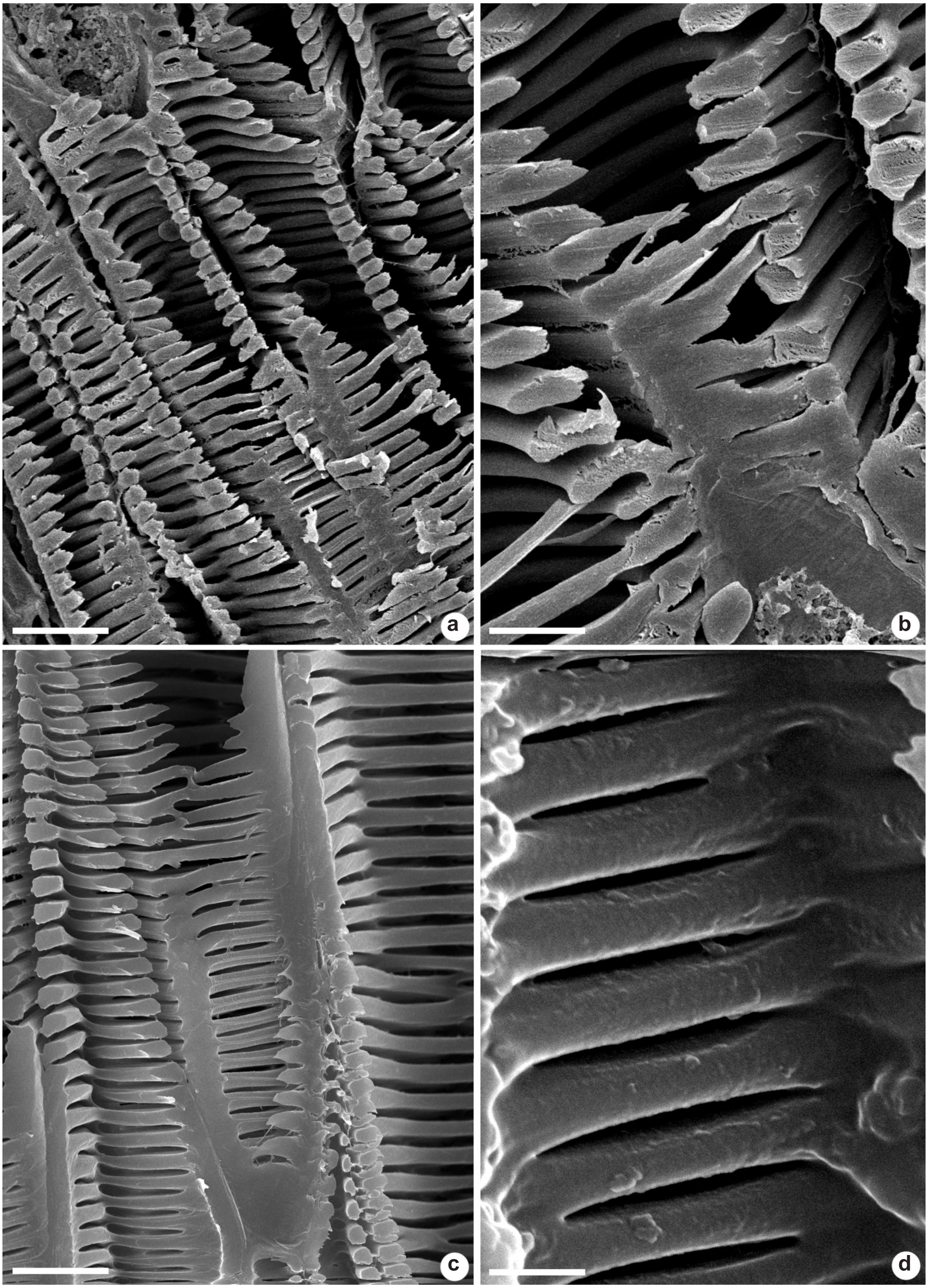

Figure 3 – a-d. Traqueary elements of Aspleniaceae species without pit membrane – a-b. *Asplenium serratum*; c. *Hymenasplenium triquetrum*; d. *A. monanthes.* Scale bars: a-b = 10µm; c-d = 2µm.

rupestral or terrestrial). Likewise, in the present work, we found no evidence of correlation between habitat and tracheary elements.

Regarding the second question, it is possible to analyze the data of the present work in the light of the phylogeny proposed by Schneider *et al.* (2004). The authors proposed the genus *Hymenasplenium* as *Asplenium*'s sister clade, which is in turn divided into eight clades. By analyzing the three species that presented vessel elements (*Hymenasplenium triquetrum*, *A. monanthes*, and *A. serratum*) and finding that the species belong to distinct groups, we can infer that vessel elements emerged several times independently within the family.

Question 3 raises the possibility that the occurrence in ferns of both end-wall and lateral-wall perforation plates has the effect of converting the fascicle of tracheary elements into a kind of aggregate vessel (Carlquist & Schneider 2001). Thus, in species in which the xylem was forming areas with aggregate tracheal elements and not intermixed with parenchyma cells, one would expect to find vessel elements. However, Wetzel *et al.* (2017), studying the anatomy of the vegetative axis of Aspleniaceae, demonstrated that xylem does not form large aggregates in species with vessel elements.

This work shows that *Asplenium* has species with elongated conducting cells with oblique end walls and scalariform thickening on all faces of the cells. The number of faces and presence of pit membrane varies between species. *H. triquetrum*, *A. monanthes* and *A. serratum* have vessel elements. *A. raddianum*, *A. unisseriale* and *A. sandicinum* have tracheids with intermittent perforation plates. The other species, the majority, have tracheids with nonporous or porous membrane. The results show that even within the same genus there can be great variation in relation to the types of transport cells. The reasons why such a variety of cell types exist remain to be clarified. Apparently, in the group, there is no relationship between the types of conductive cells and habitat or habitat type. Likewise, the idea of association between aggregated vessel elements does not make sense, as observed in results, there is no relationship between the types of conductive cells and their arrangement.

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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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