

# Fern phloem evolution records a trend of decreasing conductivity through geological time

Corresponding Author: Dr Alexander Hetherington

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Comments for the Author)

The authors demonstrated a trend in sieve element (SE) size reduction in fern lineages by measuring a vast amount of SE cell sizes in both extant and fossilized specimens (including published material and re-imaged museum samples). This is supported by ancestral state reconstruction; the SE element size retains also a phylogenetic signal supported by larger SE element size in basal nodes of phylogenetic tree.

This study is the first to include phloem tissue measurements from such a large number of species, including extinct specimens from museum collections. The results demonstrate that the vascular system has evolved over time, with changes in physiology and/or transport efficiency, likely in response to ecological niche adaptations.

Overall, this is a highly interesting paper that is well suited for the broad readership of Nature Plants. It is well-written and it presents a novel perspective on phloem evolution, integrates evidence from multiple lines of inquiry, and is supported by extensive datasets as well as robust mathematical and theoretical analyses. I have a few points that require clarification.

Specific comments

1. How do the authors confidently identify sieve elements (SEs) in the histological analyses? In particular, identification appears challenging in fossil material, such as the sample shown in Fig. 5c. If there is uncertainty in recognizing SEs in certain specimens, this should be explicitly acknowledged. The authors should provide a clear description of the criteria used to identify SEs, for example in the Methods section. This is especially important given that the central conclusions of the paper rely on the correct identification of SEs.
2. Is it possible that sieve element (SE) diameters in fossil samples have increased over the approximately 400 million years since deposition, for example due to taphonomic or diagenetic processes? If not, please explain why you think so. If there is any uncertainty related to this, it would be important to acknowledge and discuss it in the Discussion section.
3. Is Midpoint of the Petiole (MPOP) consistent across more species. The authors have showed that across two species there seems to be a consistent way of measuring the SE size. But taking account the diversity of frond shapes, would the basal part be more consistent measuring location across all species? This would also theoretically include the maximal flow rate when the leaves are a carbon source.
4. Have the authors considered adding more data from the models in the supplementary data. E.g. the residual plots would be helpful in assessing if the data would need transformations like Box-Cox or should GLMs be considered.
5. in Fig 1. is the i-l, *Thyrsopteris elegans* species picture the correct species? Just to double-check...

(Remarks on code availability)

Reviewer #2

(Comments for the Author)

While this manuscript presents interesting hypotheses, in my opinion as a plant anatomist I find the method of identifying sieve elements to be inadequate. Simply looking for small cells seemingly with out contents does not differentiate parenchyma cells from sieve elements. Location is simple not as reliable as the authors think it is. Often parenchyma cells can be intermixed with sieve elements in ferns. That is a small parenchyma cell with a vacuole in transverse sections can appear to be devoid of contents. Also, sieve cells are devoid of contents, they just appear to be so with the staining methods used by the authors. Basically, sieve cells are comprised primarily of a proteinaceous matrix as the result of the breakdown of the nucleus vacuoles and other organelles. Additionally, the cell walls will have sieve areas with callose. The latter are rarely seen in transverse sections unless an end wall is present. The cytoplasm will appear clear with the stains used in this work. Stains specifically for sieve cells with a protein matrix need to be used. Also, a stain for callose occurring around the sieve pores could be used. I suggest the following.

Sieve elements can be identified by staining for callose, the polysaccharide that is found at sieve areas, or by staining for proteins. These include aniline blue with fluorescence for callose or Toluidine Blue O that would stain the sieve elements pinkish color or an actual protein stain such as Coomassie Blue.

This only needs to be done on one section for each species to demonstrate that parenchyma cells are not present among the sieve cells. Similarly, longitudinal sections could be used to establish the presence of sieve area on lateral and end walls.

(Remarks on code availability)  
I think this is not applicable here.

Reviewer #3

(Comments for the Author)

The manuscript entitled "Phloem evolution in ferns was shaped by phylogeny and records a trend of decreasing conductivity through geological time" aimed to investigate the evolution of sieve tube elements in fern petioles. Overall, I find this work novel, unique, and worthy of study. To date, there has been no formal examination of fern phloem in an evolutionary context.

That said, I do believe that the analyses, narrative, and interpretations need more work before the manuscript is ready for publication. I aim to provide a constructive, not destructive, set of comments and suggestions that I hope will elevate the work and make it ready for publication in the future.

Specifically, I have several major points. First, the statistical framework needs to be updated to include phylogenetic integration (such as PIC or PGLS) to account for shared evolutionary history and potential pseudo-replication (e.g., multiple samples per species). Additionally, some conceptual framing regarding "phylogenetic signal" versus "shaping" forces needs adjustment, and the structural organization of the results and discussion should be tightened. For instance, I would caution against the statement that shared evolutionary history "shaped" SE radius. Rather when there is high phylogenetic signal there are two possibilities. The trait could be under relaxed selection and thus drift could be the factor of limited change and high signal. Alternatively, the trait could be highly adaptive and conserved broadly. But, regardless, the phylogeny is not "shaping" the trait. Rather the pattern is observed across the phylogeny and the processes are either drift or selection. I recommend reading: Revell, Liam J., Luke J. Harmon, and David C. Collar. "Phylogenetic signal, evolutionary process, and rate." *Systematic biology* 57.4 (2008): 591-601. Likewise, the discussion of vascular scaling may be heightened by reading and interacting with work on xylem tapering by Mark Olson and also some more work by Jessica Savage which the authors did not reference, particularly: Clerx, Laura E., et al. "Ontogenetic scaling of phloem sieve tube anatomy and hydraulic resistance with tree height in *Quercus rubra*." *American journal of botany* 107.6 (2020): 852-863. Importantly, I really worry about size of the extinct fossil SE. The micrographs don't seem to look like SE and the size of *Psaronius* SE is a bit too large (larger than some of the largest Angiosperm SE!). Lastly, while I find the work inherently very interesting and exciting, the discussion section was quite flat and devoid of analysis. It read more like a methods section at many parts. I think that the manuscript would be elevated by thinking about how their analyses fit into a broader evolutionary perspective (e.g., what evolutionary concepts and theories can explain the macroevolutionary patterns).

I just want to reiterate that I find this work interesting and worthy of study. I hope my comments and suggestions can be interpreted constructively. I hope to eventually see this manuscript in press.

Abstract

Line 19–20: I think this statement could be reframed or reworded: "phloem structure was uniform through time in ferns." What does it mean to be "uniform" through time? Is the null hypothesis or expectation that phloem is unchanged? In what ways could phloem change exactly? This is a very interesting question, but I think the excitement becomes slightly dull in the current phrasing.

Introduction

This is clean and well written. I have no suggestions for the introduction.

Results and Discussion

Line 106–108: seems like this could be moved to methods

Line 112–113: the discussing may be enhanced by integrating more of the xylem tapering (base to tip) work from Mark Olson and colleagues (also with Line 117)

Line 113: this could also be methods. I will also say, did the authors

Line 118: it seems quite odd that phloem would be largest in the middle instead of at the base.

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Line 120: I think it may be also worthwhile to examine the tapering dynamics along petioles across species. There may be some interesting scaling insights.

Line 132–133: So, it seems like the smallest leaved plants have the smallest phloem and the largest has the largest?

Line 198: “quatre” typo

Line 238: pagels lambda is typically between 0–1, are the authors certain about their 1.10 value?

Line 257: I would caution against saying that phylogeny “shaped” a trait

Line 388–390: the statement about correlations seems to contradict previous statements about the lack of correlation.

Figure 2: Could be good to indicate which samples are significantly different from one another.

Figure 3: the use of numerous individuals per species and the lack of phylogenetic correction concerns me in this figure

Figure 4: so, without Angiopteris how variable are phloem cell sizes between species? Also is a difference of ~2-5 biologically significant? I would also caution against interpretations of the common ancestor in quantitative and recons. It will always be the mean value in your trait space.

Figure 5: could those be tracheary idioblasts? They are massive! I worry about the exclusive interpretation of those as large leptoids. For instance, in panel b there are some cells to the left that look like tracheary idioblasts. I recognize that the upper cells have much thinner cell walls, but perhaps that's an artifact or a developmental dynamic. Also, the size of the sieve tube elements in *Psaronius* is a big concern. Some of the largest sieve tube elements in flowering plants are ~20-50µm or so. Having SE that are 60 seems off.

Figure 6: The authors may benefit from thinking about scaling relationships and exponents of this scaling relationship. For instance, do sieve element size scale to the X-power number or leaf size?

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Comments for the Author)

The authors have addressed my concerns well. Only issue that remains is the interpretation of the new Figure S7. To me the data shows that extant species have larger phloem cells than extinct species (i.e. phloem cells INCREASE their radius through time), rather than the other way round, as the authors interpret. Please, clarify this.

(Remarks on code availability)

I was assisted (like in the first round) with my PhD student Leo Vainio: <leo.vainio@helsinki.fi>

Reviewer #2

(Comments for the Author)

The resubmission is very well done and successfully answers the reviewer's concerns. There remains one issue that the reviewers failed to mention. This can be addressed in one paragraph in the discussion.

Given that decreased diameter of sieve cells can reduce the assimilate conduction does not mean that overall conduction is decreased. In fact, it could be enhanced. That is, perhaps the same space is occupied by more numerous additional smaller sieve cells. This would maintain total conduction and at the same time protect against injuries and embolisms. That is, if a

longitudinal series of large sieve cells is damaged or occluded then function is lost. In contrast, with several smaller series of sieve cells that damage is minimized when one of the series becomes nonfunctional. I note that extant tree ferns and lianas seem to be doing well. So perhaps if evolution is about problem solving, this may well be a good example. All of this is a testable hypothesis by conducting a similar study on other groups such as lycophytes or gymnosperms.

My suggestion is to add another paragraph for a broader context.

(Remarks on code availability)

Reviewer #3

(Comments for the Author)

Overall, I found the response to the reviews to be quite satisfactory. I have but several comments and suggestions that I will add below.

Line 23: could just remove "next" and start with "By integrating.."

Line 32: change "plant life" to "plant survival"

Line 44: I think this should be to the fourth power based on the Hagen-Poiseuille equation.

Line 59: I'd suggest tempering the phrase "transforming" perhaps, "adding to"

Line 60: I'd add a semicolon before "the lycophytes and ferns."

Fig. 6: The authors may want to examine the point on the lower right side of fig 5a, as it seems like an odd outlier

(Remarks on code availability)

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## Reviewers' Comments:

### Reviewer #1 (Comments for the Author):

The authors demonstrated a trend in sieve element (SE) size reduction in fern lineages by measuring a vast amount of SE cell sizes in both extant and fossilized specimens (including published material and re-imaged museum samples). This is supported by ancestral state reconstruction; the SE element size retains also a phylogenetic signal supported by larger SE element size in basal nodes of phylogenetic tree.

This study is the first to include phloem tissue measurements from such a large number of species, including extinct specimens from museum collections. The results demonstrate that the vascular system has evolved over time, with changes in physiology and/or transport efficiency, likely in response to ecological niche adaptations.

Overall, this is a highly interesting paper that is well suited for the broad readership of Nature Plants. It is well-written and it presents a novel perspective on phloem evolution, integrates evidence from multiple lines of inquiry, and is supported by extensive datasets as well as robust mathematical and theoretical analyses. I have a few points that require clarification.

**Response:** We are grateful that Referee 1 thought this was a highly interesting paper, and well suited to Nature Plants.

### Specific comments

1. How do the authors confidently identify sieve elements (SEs) in the histological analyses? In particular, identification appears challenging in fossil material, such as the sample shown in Fig. 5c. If there is uncertainty in recognizing SEs in certain specimens, this should be explicitly acknowledged. The authors should provide a clear description of the criteria used to identify SEs, for example in the Methods section. This is especially important given that the central conclusions of the paper rely on the correct identification of SEs.

**Response:** As recommended by Referee 1, we have made the following adjustments:

- i. For extant species, we have: Added a new criterion to identify sieve elements in our dataset, the presence of refractive spherules. Added two new supplementary figures (Fig. S1, S2) to illustrate the criteria used to identify SEs. Provided a new supplementary Table (Table S2) reviewing in detail previous descriptions of phloem in ferns and listing how these published data were used to help us identify SEs in extant species.
- ii. Added a new section to the methods (line 436 – 452) explicitly describing the criteria used to identify SEs for extant ferns:  
“Our identification of SEs in extant species is based on a summary of previously published descriptions of phloem in ferns which we summarise in Table S2. Using these descriptions, we first located phloem tissue in our samples, and then identified SEs among phloem cells using one or both of these criteria:  
1) Absence of nuclei and densely stained protoplasts (Illustrated in Fig. S1). In some cases, the protoplast of parenchyma cells can be stained lightly, but staining is always more prevalent than in SEs. This criterion was applicable for 25 of 26 species in our dataset.  
2) The presence of refractive spherules (Illustrated in Fig. S2). Refractive spherules were identified in 13 of 26 species in our dataset.

The only case where neither of these criteria was applicable was *Psilotum nudum*. However, as documented in Table S2, sieve elements were readily identified by their position within the stele and thicker cell walls than surrounding parenchyma.

Of our samples, 18 of the 26 species correspond to existing descriptions of the same species or genus within the literature. The remaining eight species belong to Polypodiales, the largest clade of ferns; for these, no matching descriptions at the genus level were found. Nevertheless, it was possible to identify their SEs by applying the two summarized criteria that hold true for the majority of fern species.”

- iii. For extinct taxa we also added a new supplementary table (Table S13) summarising published studies that were used to help us classify SEs in fossils.
- iv. We also added a new description of the method used to describe SEs in extinct taxa (line 597 – 610):

“The identification of SEs in fossils was conducted strictly according to the published literature and the criteria used for each species are listed in Table S13. In published studies, phloem and SEs were characterized using both transverse and longitudinal sections, allowing SEs to be identified as elongated cells, with tapered end walls, and sieve areas and pores. In summary, there were three scenarios for different species:

  - 1) In *Etapteris leclercqii*, *Botryopteris tridentata* and *Anachoropteris involuta*, there is no parenchyma cells in between SEs, therefore the measurements were taken entirely on SEs.
  - 2) In *Psaronius sp.* and *Stauropteris burntislandica* (according to the reference of *Stauropteris biseriate*<sup>58</sup>), both SEs and phloem parenchyma cells can be observed in phloem but they are not distinguishable from each other in transverse section where measurements should be taken. Therefore, all phloem cells were measured.
  - 3) In *Sphenophyllum plurioliatum*, *Tempskya dernbachii* (primary phloem) and *Sphallopteris schlechtendali*, it is unknown whether phloem contains parenchyma cells, therefore all phloem cells were measured.”
- v. As the reviewer noted, confidently identify SEs in fossils is challenging. As noted above to overcome this as much as possible we deferred our assignment to already published data on fossil SEs. However, in fossils there is still a risk that our dataset may include some parenchyma cells as well as SEs. To ensure that this did not have a major impact on our findings we carried out a new analysis described in methods (line 611 - 621) in which we compared the radius of all phloem cells, the combination of both phloem parenchyma (PPs) and SEs in the extant members of the Equisetales, Osmundales and Marattiales and the phloem cells (PCs) in their extinct relatives (Fig. S7). This analysis demonstrated that the inclusion of phloem parenchyma of extant species in our dataset did not change our conclusions and there remained significant differences in size between extant and extinct taxa, with radius of phloem cells being larger in extinct than extant taxa. This new analysis and supplementary figure demonstrate that our approach to compare phloem in extinct taxa was robust.

2. Is it possible that sieve element (SE) diameters in fossil samples have increased over the approximately 400 million years since deposition, for example due to taphonomic or diagenetic processes? If not, please explain why you think so. If there is any uncertainty related to this, it would be important to acknowledge and discuss it in the Discussion section.

Response: Thank you for raising this point. We think it is highly unlikely that the sieve element (SE) diameters in our material have been inflated or distorted over time for two reasons:

- i. Our dataset is based on permineralized fossils, in which the mineral matrix precipitated around plant tissues before significant burial or compaction could occur. The exceptional preservation of these samples also demonstrated that there is no evidence of taphonomic break-up or distortion of cells.
- ii. Typically, when fossils are distorted taphonomically the result is cell shrinkage (e.g. Kerp 2017 doi: 10.1098/rstb.2016.0495). Cell shrinkage may also occur when generating fossil preparations such as peels (e.g. Long et al., 10.1016/j.revpalbo.2022.104750). However, we are not aware of any reports or mechanisms that would result in cells swelling or increasing in size over time. The following text is already included in our manuscript (line 622):

“To allow comparisons between fossils and extant ferns, all the measurement taken from samples prepared with cellulose acetate peel technique were corrected by a  $\times 1.05$  factor due to the shrinkage caused by the peeling technique<sup>59</sup>”

3. Is Midpoint of the Petiole (MPOP) consistent across more species. The authors have showed that across two species there seems to be a consistent way of measuring the SE size. But taking account the diversity of frond shapes, would the basal part be more consistent measuring location across all species? This would also theoretically include the maximal flow rate when the leaves are a carbon source.

Response: We chose the MPOP over the base of the petiole because of the following reasons:

- The cellular structure in the base of the petiole can be easily destroyed during sampling.
- The base of petiole usually contains more complicated vascular architecture, e.g., a larger number of vascular bundles, compared with that in MPOP (Ogura, 1972; Brodersen *et al.*, 2012).
- We expect flow rate along the petiole to be relatively constant because almost all photosynthesis is taking place in the leaf blade.

We therefore deem the MPOP as the best consistent sampling point across highly disparate taxa.

4. Have the authors considered adding more data from the models in the supplementary data. E.g. the residual plots would be helpful in assessing if the data would need transformations like Box-Cox or should GLMs be considered.

Response: These have been added and are now included as Fig. S5.

5. in Fig 1. is the i-l, *Thyrsopteris elegans* species picture the correct species? Just to double-check...

Response: We thank reviewer 1 for spotting this. The species name now has been corrected as *Dicksonia antarctica*. All the related analysis and documents have been updated with the correct name.

Reviewer #2 (Comments for the Author):

While this manuscript presents interesting hypotheses, in my opinion as a plant anatomist I find the method of identifying sieve elements to be inadequate. Simply looking for small cells seemingly with empty contents does not differentiate parenchyma cells from sieve elements. Location is simply not as reliable as the authors think it is. Often parenchyma cells can be intermixed with sieve elements in ferns. That is a small parenchyma cell with a vacuole in transverse sections can appear to be devoid of contents. Also, sieve cells are devoid of contents, they just appear to be so with the staining methods used by the authors. Basically, sieve cells are comprised primarily of a proteinaceous matrix as the result of the breakdown of the nucleus, vacuoles and other organelles. Additionally, the cell walls will have sieve areas with callose. The latter are rarely seen in transverse sections unless an end wall is present. The cytoplasm will appear clear with the stains used in this work. Stains specifically for sieve cells with a protein matrix need to be used. Also, a stain for callose occurring around the sieve pores could be used. I suggest the following.

Sieve elements can be identified by staining for callose, the polysaccharide that is found at sieve areas, or by staining for proteins. These include aniline blue with fluorescence for callose or Toluidine Blue O that would stain the sieve elements pinkish color or an actual protein stain such as Coomassie Blue.

This only needs to be done on one section for each species to demonstrate that parenchyma cells are not present among the sieve cells. Similarly, longitudinal sections could be used to establish the presence of sieve areas on lateral and end walls.

Response: We thank reviewer 2 for their comments. Reviewer 1 also requested more information about how we identified SE in ferns. We have responded to the point by Reviewer 1 above, but to briefly summarise again. Based on these comments we have greatly expanded the justification of assignment of SEs in extant species. To briefly summarise, we have:

- Added a new criterion to identify SEs in our samples, the presence of refractive spherules.
- Added two new supplementary figures illustrating the criteria used (Fig. S1, S2).
- Provided a new extensive supplementary table (Table S2) which includes a detailed review of previously published studies of fern SEs used to help identify SEs in our samples.
- We have updated the methods section to state our approach for SE identification more explicitly.

Reviewer 2 has requested that we carry out a further analysis of all ferns investigated, including new histological analyses with both transverse and longitudinal sections and stains for protein matrix and stains for callose. The aim for this is to provide more confidence in our assignment of SEs, and our ability to distinguish between parenchyma and SEs. We feel this is beyond the scope of the current study because these techniques have already been carried out by previous authors investigating fern phloem anatomy. Rather than repeating this extensive body of work we have instead summarised these findings in our new Table S2. Data provided in Table S2 documents previous investigations on the major fern families using the techniques requested by Reviewer 2. These include detailed histological investigation with both transverse and longitudinal sections and stains for proteins and callose. We used the results of these previous studies to inform the criteria used in our current analysis and to provide extra confidence that we could distinguish SEs from parenchyma using two key criteria already observable in our current dataset (1) The absence of nuclei and absence of densely stained protoplasts, and (2) the presence of refractive spherules are SEs.

Reviewer #3 (Comments for the Author):

The manuscript entitled “Phloem evolution in ferns was shaped by phylogeny and records a trend of decreasing conductivity through geological time” aimed to investigate the evolution of sieve tube elements in fern petioles. Overall, I find this work novel, unique, and worthy of study. To date, there has been no formal examination of fern phloem in an evolutionary context.

That said, I do believe that the analyses, narrative, and interpretations need more work before the manuscript is ready for publication. I aim to provide a constructive, not destructive, set of comments and suggestions that I hope will elevate the work and make it ready for publication in the future.

Specifically, I have several major points. First, the statistical framework needs to be updated to include phylogenetic integration (such as PIC or PGLS) to account for shared evolutionary history and potential pseudo-replication (e.g., multiple samples per species).

Response: We have made the following update to our statistical framework:

- i. PGLS is used as suggested.
- ii. Species level data instead of individual data has been used to avoid pseudo-replication. We were able to include an additional 11 species by adjusting our analysis framework to the species level rather than the individual level, allowing the inclusion of 23 species in total in our models including leaf data.
- iii. Related main text and methods have been rewritten according to the results of PGLS.

We are very grateful for this comment, this has greatly strengthened our results and manuscript.

Additionally, some conceptual framing regarding "phylogenetic signal" versus "shaping" forces needs adjustment, and the structural organization of the results and discussion should be tightened. For instance, I would caution against the statement that shared evolutionary history “shaped” SE radius. Rather when there is high phylogenetic signal there are two possibilities. The trait could be under relaxed selection and thus drift could be the factor of limited change and high signal. Alternatively, the trait could be highly adaptive and conserved broadly. But, regardless, the phylogeny is not “shaping” the trait. Rather the pattern is observed across the phylogeny and the processes are either drift or selection. I recommend reading: Revell, Liam J., Luke J. Harmon, and David C. Collar. "Phylogenetic signal, evolutionary process, and rate." *Systematic biology* 57.4 (2008): 591-601.

Response: We thank reviewer 3 for raising this point – this is a very constructive suggestion. We have removed previous statements that the phylogeny “shaped” or was a “shaping” force and instead now state that “phylogenetic structured pattern across species”.

We have also added a new section “The role of scaling relationships in fern phloem evolution” (line 314) to discuss the evolutionary pattern suggested by high phylogenetic signal and strong correlations.

Finally, we have also integrated the findings from Revell *et al.* to our current interpretation of high phylogenetic signal in our discussion. The new text reads “Regardless of the precise impact, it is clear that under such constraints, the phylogenetic signal in SE radius may be high, in line with the pattern described by Revell *et al.*<sup>45</sup>.”

Likewise, the discuss of vascular scaling may be heightened by reading and interacting with work on xylem tapering by Mark Olson and also some more work by Jessica Savage which the authors did not reference, particularly: Clerx, Laura E., et al. "Ontogenetic scaling of phloem sieve tube anatomy and hydraulic resistance with tree height in *Quercus rubra*." *American journal of botany* 107.6 (2020): 852-863.

Response: Done. We have expanded the discussion to cite relevant work by Olson and colleagues on conduit tapering, and additional work by Savage and co-workers on height-dependent phloem scaling, including Clerx et al. (2020).

Importantly, I really worry about size of the extinct fossil SE. The micrographs don't seem to look like SE and the size of *Psaronius* SE is a bit too large (larger than some of the largest Angiosperm SE!).

Response:

The sizes and descriptions of fossil sieve elements are based on previously published work. To make this primary literature more accessible we have summarised it in a new Table (Table S8), and updated the description of our assignment of fossil SEs in the methods – as document above in response to the comment by Reviewer 1. This new text and table should provide more explicit information about how we and previous authors identified phloem in the fossil record.

In response to the point about the large size of *Psaronius*. In our dataset, the medium radius of phloem cells in *Psaronius* were 15.47  $\mu\text{m}$ , which is consistent with those reported by Smoot (1984). We agree that there are some very large cells in *Psaronius* and predicted that they may bias our results. To test this, we already included analyses where we performed our comparison excluding the very large cells (Table S10). Excluding these data did not change our conclusions.

Although cells of *Psaronius* are very large we think that their large size alone is enough to rule out their inclusion in our study. In a recently published investigation of phloem evolution from Devonian fossils (Cooper and Hetherington 2026, DOI: 10.1016/j.cub.2026.05.072) we also observed that the food conducting cells of extinct taxa were significantly larger than those of extant species. In keeping with the overall findings of this study, we predict that during vascular plant evolution there has likely been a trend of decrease in phloem cell size through time.

Lastly, while I find the work inherently very interesting and exciting, the discussion section was quite flat and devoid of analysis. It read more like a methods section at many parts. I think that the manuscript would be elevated by thinking about how their analyses fit into a broader evolutionary perspective (e.g., what evolutionary concepts and theories can explain the macroevolutionary patterns).

Response: We have incorporated this suggestion to our discussion on the PGLS analysis and interpretation of high phylogenetic signals of SE radius. We have added a new section "The role of scaling relationships in fern phloem evolution" (line 314) to discuss the possible evolutionary scenario.

I just want to reiterate that I find this work interesting and worthy of study. I hope my comments and suggests can be interpreted constructively. I hope to eventually see this manuscript in press.

Response: We thank Reviewer 3 again for their constructive comments on our study.

## Abstract

Line 19–20: I think this statement could be reframed or reworded: “phloem structure was 20 uniform through time in ferns.” What does it mean to be “uniform” through time? Is the null hypothesis or expectation that phloem is unchanged? In what ways could phloem change exactly? This is a very interesting question, but I think the excitement becomes slightly dull in the current phrasing.

Response: The word “uniform” was chosen specifically as this was a direct quote from a previous investigation.

## Introduction

This is clean and well written. I have no suggestions for the introduction.

## Results and Discussion

Line 106–108: seems like this could be moved to methods

Response: Done.

Line 112–113: the discussing may be enhanced by integrating more of the xylem tapering (base to tip) work from Mark Olson and colleagues (also with Line 117)

Response: Done

Line 113: this could also be methods. I will also say, did the authors

Response: Done

Line 118: it seems quite odd that phloem would be largest in the middle instead of at the base.

Response: The referee isn't suggesting a change here.

Line 120: should also be methods

Response: Done

Line 120: I think it may be also worthwhile to examine the tapering dynamics along petioles across species. There may be some interesting scaling insights.

Response: This is an interesting idea. However, we don't feel that its absence from the paper detracts from our overall analysis or conclusions so we feel it is beyond the scope of our current study.

Line 132–133: So, it seems like the smallest leaved plants have the smallest phloem and the largest has the largest?

Response: Yes. The referee isn't suggesting a change here.

Line 198: “quatre” typo

Response: The original text was deleted.

Line 238: pagels lambda is typically between 0–1, are the authors certain about their 1.10 value?

Response: Yes, we are sure that the value is 1.10. While lambda is typically between 0–1, a value larger than 1 is technically possible.

Line 257: I would caution against saying that phylogeny “shaped” a trait

Response: This has been changed to:

“Taken together our investigation of SE radius in a phylogenetic context highlights that evolutionary history, alongside leaf morphology and vascular architecture, is a strong predictor of SE radius.”

Line 388–390: the statement about correlations seems to contradict previous statements about the lack of correlation.

Response: The text has been deleted.

Figure 2: Could be good to indicate which samples are significantly different from one another.

Response: To do pairwise post hoc test, usually Dunn test is used. However, because many species were represented by only three individuals, and some by only a single individual, Dunn’s test had limited power for our datasets. Therefore, to confirm the significant interspecific differences were not caused by extreme values, we performed KW test with a dataset excluding a species with the largest values, *Angiopteris evecta*, and a dataset excluding both *Angiopteris evecta* and the species with the second largest values, *Osmunda regalis* (Table S4).

We think adding this information to the text figure or main text would provide too much extra noise, so we have added this information to our method (line 527 - 533):

“Because many species were represented by only three individuals, and some by only a single individual, Dunn’s test had limited power for pairwise post hoc comparisons. Therefore, to confirm the significant interspecific differences were not caused by extreme values, we performed KW test with a dataset excluding a species with the largest values, *Angiopteris evecta*, and a dataset excluding both *Angiopteris evecta* and the species with the second largest values, *Osmunda regalis* (Table S4). SD and CV were calculated accordingly (Table S5).”

Figure 3: the use of numerous individuals per species and the lack of phylogenetic correction concerns me in this figure

Response: Correction is done. The analysis has been replaced with PGLS analysis. Species mean values are used now to replace multiple individuals per species in previous LMM models.

Figure 4: so, without *Angiopteris* how variable are phloem cell sizes between species?

Response: We have already responded to this above.

Also is a difference of ~2-5 biologically significant?

Response: Based on our modelling data described in the discussion a difference of between 2-5 is predicted to result in a about more than two times difference in phloem conductivity. Here is our original text in the discussion (line 289 – 294):

“Finally, even when solely focusing on data of living species, we predicted using ASR that there was a decrease in SE radii from the common ancestors of all living ferns (Fig. 4 a, SE radius = 5.5  $\mu\text{m}$ ) to the common ancestors of eupolypods (Fig. 4 e, SE radius = 3.4  $\mu\text{m}$ ). Although data from living species suggest only a 2.1  $\mu\text{m}$  decrease in SE radii, this still accounts for a more than two-fold decrease in conductivity through fern evolution.”

I would also caution against interpretations of the common ancestor in quantitative and recons. It will always be the mean value in your trait space.

Response:

Quantitative ancestral state reconstruction is a valuable approach to make predictions about character evolution through time. Especially in this context when we explore these predictions further in fossils later. Here this approach helped us identify that most of the extant non-osmundaceous leptosporangiate ferns and the estimated ancestral values in this clade exhibit smaller SE radius compared to that of the common ancestor of all ferns.

However, we understand the concern raised by the reviewer and had considered it in our writing. For example, we state there is a “general shift towards smaller SE radii in non-osmundaceous leptosporangiates” rather than a trend towards smaller SE radii in all ferns.

Figure 5: could those be tracheary idioblasts? They are massive! I worry about the exclusive interpretation of those as large leptoids. For instance, in panel b there are some cells to the left that look like tracheary idioblasts. I recognize that the upper cells have much thinner cell walls, but perhaps that's an artifact or a developmental dynamic. Also, the size of the sieve tube elements in *Psaronius* is a big concern. Some of the largest sieve tube elements in flowering plants are ~20-50  $\mu\text{m}$  or so. Having SE that are 60 seems off.

Response: We have already responded to this above in the major points from the referee.

Specifically for *Psaronius* in previous descriptions (Smoot EL. 1984. Phloem structure in the Carboniferous fern *Psaronius* (Marattiales). American Journal of Botany 71: 1104–1113.), the large cells range 90 to 120  $\mu\text{m}$  in diameter, which matches our measurements. Based on longitudinal sections, the author described that these large cells are elongated and “the lateral and end walls contain perforations that are comparable in size and shape to those present on the smaller sieve elements.” Based on these descriptions, we considered these cells as sieve elements.

We did consider the possibility of these very large cells could potentially introduce bias into our results. Therefore, we had performed the tests with a dataset excluding all the very large cells (Table S10). Excluding these data did not change our conclusions.

In conclusion, we based our descriptions on those published in the literature, where large sizes had been found and sieve element characteristics were observed. However, if the very largest of these is another tissue and were removed from our analysis the same conclusions hold as documented in Table S10.

Figure 6: The authors may benefit from thinking about scaling relationships and exponents of this scaling relationship. For instance, do sieve element size scale to the X-power number or leaf size?

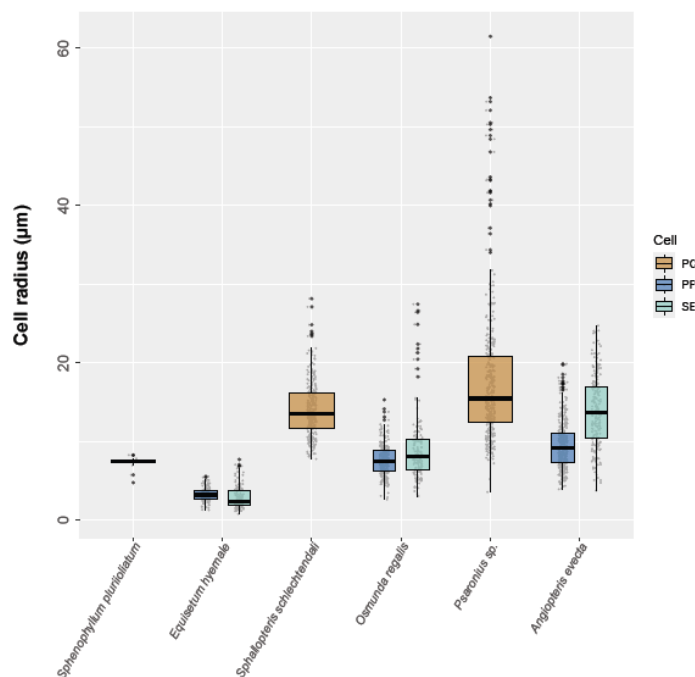
Response: We thank the reviewer for this insightful suggestion. We agree that investigating scaling exponents could provide additional insight into the relationships between sieve element size and leaf traits. However, our primary objective in this study was to establish whether these traits are associated across species rather than to characterize the underlying scaling laws.

The PGLS models presented in the manuscript already demonstrate strong relationships, with high  $R^2$ , supporting our main conclusions. A comprehensive analysis of scaling exponents would require additional methodological considerations and would substantially expand the scope and length of the manuscript. We therefore consider this an interesting direction for future work rather than an essential component of the present study.

Reviewer #1 (Comments for the Author):

The authors have addressed my concerns well. Only issue that remains is the interpretation of the new Figure S7. To me the data shows that extant species have larger phloem cells than extinct species (i.e. phloem cells INCREASE their radius through time), rather than the other way round, as the authors interpret. Please, clarify this.

Response: We thank Reviewer 1 for spotting this mistake. The error resulted from a mistake in our code where we square-rooted data for extinct species twice resulting in these values appearing smaller than they should have been. We have corrected the figure and as already stated in the manuscript the extinct species have larger phloem cells than those in extant species, which is consistent with our overall narrative.



Reviewer #2 (Comments for the Author):

The resubmission is very well done and successfully answers the reviewer's concerns. There remains one issue that the reviewers failed to mention. This can be addressed in one paragraph in the discussion.

Given that decreased diameter of sieve cells can reduce the assimilatory conduction does not mean that overall conduction is decreased. In fact, it could be enhanced. That is, perhaps—the same space is occupied by more numerous additional smaller sieve cells. This would maintain total conduction and at the same time protect against injuries and embolisms. That is, if a longitudinal series of large sieve cells is damaged or occluded then function is lost. In contrast, with several smaller series of sieve cells that damage is minimized when one of the series becomes nonfunctional. I note that extant tree ferns and lianas seem to be doing well. So perhaps if evolution is about problem solving, this may well be a good example. All of this is a testable hypothesis by conducting a similar study on other groups such as lycophytes or gymnosperms.

My suggestion is to add another paragraph for a broader context.

Response: This is a very good point raised by Reviewer 2, but is one already covered in our revised manuscript. In lines 299-312 we specifically describe that small size is not accounted for by larger number of cells. Instead, the converse is true:

“Our method was focussed on sampling average SE radius in each species. However, when considering overall phloem transport capacity, it is the total summed area of all SEs that would dictate flow. There is therefore a chance that species having smaller SE radii were compensated with larger number of SEs. We tested this by quantifying both the size and number of SEs. In extant species, we identified a positive correlation between SE radius and SE number (Fig. 6a,  $t = 2.686$ ,  $df = 24$ ,  $p\text{-value} = 0.013$ , Pearson's  $r = 0.481$ ), indicating that species with larger SE radii usually also have more SEs. A few species may be outliers, for example *Psilotum nudum*, which lies in the lower right of the plot. However, these species did not alter the overall trend. This suggests a double penalty on phloem transport capacity in non-osmundaceous leptosporangiate ferns, with a simultaneous decrease in both SE radius and number leading to a net reduction in total conducting area. Consistent with lack of a compensatory mechanism involving the development of more SEs to compensate for small radius we identified that in fossils with large SE radii that they had no significant difference in SE number compared to extant species (Fig. 6b,  $\chi^2 = 0.014$ ,  $df = 1$ ,  $p\text{-value} = 0.903$ ). These finding further support the prediction of a predicted decrease in conductivity through fern evolution.”

As this text was already in the manuscript, we have not made any additional revisions.

Reviewer #3 (Comments for the Author):

Overall, I found the response to the reviews to be quite satisfactory. I have but several comments and suggestions that I will add below.

Line 23: could just remove “next” and start with “By integrating..”

Response: done.

Line 32: change “plant life” to “plant survival”

Response: done.

Line 44: I think this should be to the fourth power based on the Hagen-Poiseuille equation. The referee is correct that the Hagen-Poiseuille flow rate scales with the fourth power of the radius. However, we meant to convey the relation between flow speed and conduit dimensions. The flow speed scales with the square of conduit radius. Therefore in the manuscript, we have corrected the text from “flow rate” to “flow speed” in lines 43 and 48.

Line 59: I’d suggest tempering the phrase “transforming” perhaps, “adding to”

Response: done.

Line 60: I’d add a semicolon before “the lycophytes and ferns.”

Response: We have used a colon instead, as it is more grammatically appropriate. However, we are happy to follow journal style and the editor’s decision here.

Fig. 6: The authors may want to examine the point on the lower right side of fig 5a, as it seems like an odd outlier

Response: To highlight this as outlier we have made this change to the text. Line 252:

“In extant species, we identified a positive correlation between SE radius and SE number (Fig. 6a,  $t = 2.686$ ,  $df = 24$ ,  $p\text{-value} = 0.013$ , Pearson's  $r = 0.481$ ), indicating that species with larger SE radii also **tend to** have more SEs. **A few species may be outliers, for example *Psilotum nudum*, which lies in the lower right of the plot. However, these species did not alter the overall trend.** This suggests a double penalty on phloem transport capacity in non-osmundaceous leptosporangiate ferns, with a simultaneous decrease in both SE radius and number leading to a net reduction in total conducting area.