Leaf water relations in epiphytic ferns are driven by avoidance rather than tolerance mechanisms

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Running Head: Terrestrial and epiphtic fern functional traits

# Abstract

Key Words:

# Introduction

* Paragraph: epiphytism, ferns and evoln. history
  + Following the rise of angiosperms, a major radiation event in ferns occurred in the Cretaceous period, leading to diversification and proliferation of modern fern taxa (Schuettpelz & Pryer, 2009). Globally, fern species continue to adapt and thrive in shaded understory environments. Part of this recent Cretaceous radiation event in ferns (polypods), however, included oppourtunistic diversification into more complex ecological niches provided by now dominant angiosperm canopies (Schneider *et al.*, 2004). Consquently, one clade of ferns (Euploypoid I) has largely radiated into epiphytic niches, while a sister clade (Eupolypoid II) remained and diversified on the forest floor in tropical ecosystems (Watkins & Cardelús, 2012).
* Paragraph: microhabitat differences
  + terrestrial vs epiphytes (cardelus)
  + Within forests, epiphytes grow at many levels. Some are restricted to the dark understory, whereas others grow on the exposed twigs of emergent tree (Watkins & Cardelús, 2012).
  + As non-parasites, epiphyitc plants face a variety of ecological stressors, regardless of canopy position, that drive selection of functional traits key across their life history.
  + hemi-epiphyitc life form co-exists with both these groups which may represent an evolutionary transition between terrestrial and epipythic life histories.
* Paragraph: Consequences of radiation of plants into canopy, Evolution of traits
  + A host of studies reveal how anatomical traits directly influence physiology in ferns in a manner that is functionally different from seed plants (Woodhouse & Nobel (1982), Zhang *et al.* (2009), Brodersen *et al.* (2012), Brodribb *et al.* (2005)).
  + includes drought tolerance in gametophyte life stage, in both life stages [watkins]
  + It appears likely that the canalization of stomatal traits…
  + Regardless, epiphytic ferns span a vast arraw of micro-habitats that will undergo a suite of environmental stresses (high light, high pH, limited water) that should….
* Paragraph: water relations and structure
  + As water transport in ferns occurs exclusively through primary vascular tissue, wrapped in discrete bundles that can span the entire frond (Pittermann *et al.*, 2011)…
  + Terrestrial ferns may have reduced water use efficiency, which would be consistent with Brodribb and McAdam’s hypothesis of inefficient stomatal control.
  + if stomata are regulated by leaf water status, what traits related to water relations allows ferns to succeed. +The evolution of xylem and that of stomata are tightly interconnected (Sperry, 2004). (read) -
* Paragraph: this study seeks…
  + looking within E1 and E2 at traits related to water relations
  + hemi as intermediaries? hemi-epiphytic species begin life as epiphytes and transform into hemi-epiphytes by eventually developing terrestrial root systems (Moffett, 2000). Add watts new paper.

# Methods

## Study Site and Species Selection

The sites used for this study included two Costa Rican wet tropical forest locations at La Selva Biological Research Station in Heredia (84⁰00’12W, 10⁰25’52N) and Las Cruces Research Station in San Vito (8° 47′ 7” N, 82° 57′ 32” W). The La Selva site is a low elevation (ca 50 m) tropical forest, with a moderate dry season. The Las Cruces site is a premontane tropical forest located at a higher elevation (ca 1200 m). Both sites receive approximately 4m of annual rainfall (Holdridge, 1967, p. @gentry\_four\_1993).

A survey of morphological, stoichiometric, anatomical and leaf water relations parameters were conducted for six individuals from 39 fern species across three fundamentally distinct life forms (Table 1). Across both sites, 18 terrestrial, 15 epiphytic and 6 hemi-epiphytic species were collected and measured. In this study, terrestrial life forms were all collected from shaded closed canopy understories in the forest floor. Epiphytic life forms were sampled from trunks or within tree canopies, depending on the species. Epiphytic species were collected from canopy trees using single-rope climbing techniques. Hemi-epiphytic species were all collected along lower sections of trees trunks (1-3 m). Importantly, all sampled hemi-epiphytic species are known to have root connections to forest floor soils at some point in their life history. Individuals of species were collected across multiple populations but within similar micro-habitat conditions. Most sampled fern species, with the exception of hemi-epiphytes, were restricted to the eupolypod I and II clades. Vouchers for each species were deposited at the respective site of collection at either the La Selva (LSCR) or Las Cruces (LCCR) herbariums.

## Plant Material

Two complete fronds from sampled individuals were field collected in the early morning (6-7:00 am). One frond from each individual was utilized for pressure volume curves, while the other was sampled for structural morphology, lamina stoichmetry and anatomical traits. Stipes were cut at the base of the rhizome and cut ends were wrapped in wet paper towels and transported to the lab in black plastic bags. Stipes were re-cut under water and re-hydrated until time of hydraulic measurement (1-6 hours). Due to the difficulty in sampling some high canopy species; whole epiphytic individuals were carefully removed, maintained overnight in well-watered conditions in an ambient air laboratory and sampled the following day.

## Leaf Morphometric traits

Stipe length (cm) and lamina length (cm) were calculated from one sampled frond per individual. Total frond length was calculated as the sum of stipe and lamina lengths. Total lamina area for each frond was measured with a Li-3100 leaf area meter (LiCor Biosciences, Lincoln, NE, USA). Leaf mass per unit area (LMA, g cm-2) was calculated using the tissue punch method. For each individual, ten lamina punches (5 mm2) were dried to a constant mass and LMA was calculated as the total dry mass divided by the total area of all leaf punches.

## Anatomical traits

Stomatal density (SD) was measured by directly counting stomata on the abaxial leaf surface under 40x magnification. Three leaf punches (4 mm2 diameter) were sampled across random locations on different pinnae from each individual. The number of stomata in each field of view were counted in three random regions on each of three leaf punches. The stomatal density (# mm2) for each individual is presented as the mean SD across all 9 sampled regions. Individual images of stomata were directly photographed under 40x magnification (AmScope FMA050) across all three leaf punches per individual. Stomatal length (mm) and width (mm) of both guard cells were calculated for 9 stomata for each individual using Image J. Stomatal size (SS, mm2) was calculated as guard cell length multiplied by the combined width of each guard cell pair, as in Franks & Beerling (2009).

Stipes measured for vulnerability to cavitation were transversely sectioned by hand using razor blade. Stipes were sectioned at the distal end, where conduit resistivity impacts hydraulic conductivity the most. Sections were stained in toluidine blue and then mounted in glycerine on microscope slides. Each section was photographed using a light microscope mounted with a digital camera (Amscope FMA050). Section photographs were anatomically analyzed using ImageJ (National Institutes of Health, Bethesda, ML, USA). Total xylem vascular area (mm2) was considered the conduit lumen area and the wall area for all xylem in each cross section.

## Foliar chemistry

Sub-samples of foliage tissue used for lamina area calculation were collected across multiple pinnae for each individual. These sub-samples were dried to a constant mass and ground using a Wig-L-Bug (Sigma-Aldrich Co. St. Louis, USA). Nitrogen content and deltaC13 were measured using a Delta V isotope ratio mass spectrometer interfaced to a NC2500 elemental analyzer (Thermo Scientific) and corrected by comparison with certified standards.

Lamina chlorophyll content was determined on three different pinnae for each individual. Single point measurements of chlorophyll content (mg m-2) were measured within a 3 mm diameter circle with the CCM-300 chlorophyll content meter (Opti-Sciences, Hudson, NH, USA). Chlorophyll content per individual is expressed as the mean of point measurements across the entire frond.

## Pressure-volume relations

Tissue-water relations were determined with pressure–volume analysis (Tyree & Hammel, 1972) on fully expanded fronds with a Scholander pressure bomb (PMS Instruments Co., Corvallis, OR, USA). For each pressure-volume (PV) curve we sampled top most intact pinnae after full re-hydration. We generated pressure–volume curves by taking sequential water potential measurements (leaf) as fronds air dried, first in closed plastic bags (0-2 hrs), and then in open bags. The fresh mass was recorded immediately before and after each determination. Following each PV curve, foliar samples were dried to a constant mass to calculate relative water content (RWC).

For each PV curve, we graphed the relationship between 1/leaf and leaf mass to estimate parameters related to leaf turgor and bulk tissue water relations. We then calculated leaf water potential at turgor loss (tlp), the osmotic potential at full tissue hydration (o), the bulk modulus of tissue elasticity () and tissue capacitance (C) according to Sack *et al.* (2011).

## Statistical analysis

Linear mixed-effect models were used to test responses of functional traits to categorical fixed effects of life form and site, as well as to test for bivariate relationships among key functional traits. The interaction between life form and site was tested to confirm any potential environmental or climate influence on functional trait patterns. Generally, there were few life form x site interactions, so models with life form and site as main effects were compared to full models (AIC scores) and the most parsimonious model was selected. To test for broad differences among life forms, individual plant species were treated as random effects in each model. Tukey’s post-hoc test were performed in conjunction with ANOVA to determine which mean values of functional traits were different among fixed effect treatments with the ‘multcomp’ package (**???**). We utilized a type 3 ANOVA due to an unbalanced design with the limited number of hemi-epiphytes species available. If interactions were present, we computed pairwise comparisons with the ‘emmeans’ package (Lenth, 2018) to investigate interactions between life form and site. **For mixed-effect models, the conditional and marginal r2 values were calculated as per Nakagawa & Schielzeth (2013). Confidence intervals (95%) of linear mixed-effects models were generated using bootstrapping methods with 999 simulations, using the ‘bootMer’ function in the ‘lme4’ package (Bates et al. 2015).** Tests of differences in slopes and elevations of bivariate relationships between morphological traits were implemented using standardized major axis regression in the ‘smatr’ package in R (Warton *et al.*, 2012).

A phylogenetic tree for these 39 fern species was constructed using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992) and all evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree with the highest log likelihood (-7696.70) was selected. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 64.89% sites). The analysis involved 40 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1131 positions in the final dataset.

The phylogenetic signal (K-statistic) based on Brownian motion-based metrics was calculated for each continuous trait using the ‘picante’ package in R(Kembel *et al.*, 2010). Values of ‘K’ for a given trait that are below 1 mean that relatives resemble one another less than we would expect for Brownian motion (lower phlogenetic signal), and vice verse for values of ‘K’ above 1 (Blomberg *et al.*, 2003). Signifcant *P* values for ‘K’ indicates that close relatives are more similar than random species pairs for the given trait. Additionally, bivariate relationships among mean species values of continuous traits were analysed with phylogenetically independent contrasts (PIC) in the ‘ape’ package in R (Paradis & Schliep, 2019) to correct for possible non-independence resulting from phylogeny (Felsenstein, 1985). All PIC analyses were directly compared with the results of conventional ANOVA (described above) to better understand the functional and evolutionary relationships between traits. All tests of statistical significance were conducted at an level of 0.05 and all analyses were performed with R 3.6.0 (Team, 2013).

# Results

## Frond morphology and anatomy

Functional shifts in frond structural relationships for epiphtic species likely represent adaptation to the reduce the path length of water transport to lamina tissues. Total frond length was reduced by 26 % in epiphytic (59±2.5) compared to terrestrial (85±3.3) species, with frond length of hemi-epiphytes an intermediate between both groups (*P* = 0.021). The reduction in total frond length was driven by a large reduction (-54 %) in stipe length in epiphytic compared to terrestrial species (*P* = 0.001, Figure 1A). The majority of epiphytic and hemi-epiphytic ferns had stipes restricted to less than 20 cm (80% quantiles), while maximum stipe length of terrestrial ferns from the same probablity distribution was 42.3 cm. Allometric relationships between stipe length and lamina area were functionally different between epiphytic species compared to terrestrial and hemi-epiphytic species (Figure 1B, stipe length x life form; *P* = 0.002). Log relationships of stipe length and lamina area were positively correlated for each life form, however, pair wise differences were detected in both the slopes (*P* < 0.0001) and elevation (*P* < 0.0001) of this structural relationship for epiphytic species. Phylogenetic independent constrasts support the postive relationship between stipe length and lamina area for measured species (*P* < 0.001, r2 = 0.46).

Total xylem area was 60 % smaller in epiphytic and hemi-epiphytic species compared to terrestrial species (*P* = 0.003, Figure 2A). Consequently, terrestrial species produced more xylem area per unit leaf area than epiphytic or hemi-epiphytic species, resulting in higher Huber values (*P* = 0.001). (**add means here if no figure**). Increases in stipe length were postively correlated with increases in total xylem area across all life froms (*P* = 0.0002). Pair-wise differences were detected for the slopes (*P* < 0.0001) of this postive relationship between stipe length and xylem area across life forms, while elevations were similar (Figure 2B). Phylogenetic independent constrasts support the postive relationship between xylem area and stipe length for measured species (*P* = 0.0004, r2 = 0.27).

No differences were detected in total lamina area between any of the life form groups, due to the large amount of variation in lamina area across species (R2 marginal = 0.17 & R2 conditional = 0.89). However, leaf mass per unit area (LMA) was 67% higher in epiphytic compared to terrestrial species, with hemi-epiphytic species intermediates between both life forms (*P* = 0.002, Figure 1C). Broadly, leaf thickness of terrestrial and hemi-epiphytic ferns was constrained to less than 300 g m-2, while epiphytic ferns species were capable of much higher values.

## Lamina chemistry

Lamina nitrogen content (*N*) was 29.8% lower in epiphytic ferns compared to terrestrial and hemi-epiphytic ferns (*P* = 0.007). *N* was negatively correlated with increass in LMA for terrestrial and hemi-epiphytic species, but not for epiphytic species (LMA x life form *P* < 0.0001). Slopes and elevations of the signficant relationship between *N* and LMA varied across life forms (Figure 3A). Phylogenetic independent constrasts, however, did not detect a relationship between *N* and LMA. Additionally, lamina 13C for terrestrial and hemi-epiphytic species was more negative that epiphytic species (*P* = 0.004, Figure 2C). Lamina 13C for fern species at the higher elevation Las Cruces site was also less negative (-32.6 &permil) than fern species at the low elevation La Selva site (34.0 &permil,*P* = 0.015).

Total chlorophyll content was similar between terrestrial and epiphytic species, although epiphytic species had a lower range of chlorophyll content. Hemi-epiphytic species had similar chlorophyll content to terrestrial species, but were 37 % higher than epiphytic species (\*P = 0.031).

## Stomatal anatomy

Epiphytic and hemi-epiphytic species had 51% lower stomatal density (36±1.7 mm-2) compared to terrestrial species (72±3.1, *P* < 0.001). Stomatal length was similar across all species, however, guard cell width differed across life forms. Mean width of individual guard cells was 18 % smaller in terrestrial species compared to hemi-epiphytic of epiphytic species. Overall, stomatal size of terrestrial and epiphytic species were statistical similar, yet epiphytes had broadly higher stomatal size. Stomatal size in hemi-epiphtic species was 27 % larger than terrestrial or epiphytic life forms (*P* = 0.044). Increases in stomatal density were negatively correlated increases in stomatal density across all life forms (*P* < 0.0001). Pair-wise differences across life forms were detected for the slopes (*P* < 0.0001) and elevations (*P* = 0.007) of the negative relationship between stomatal density and stomatal size (Figure 4B).

## Frond hydraulic traits

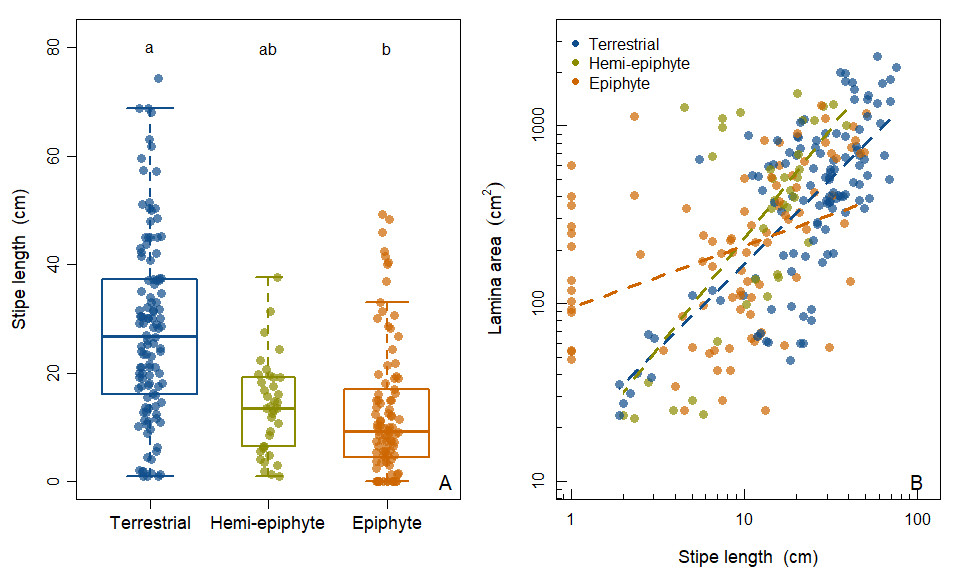
Surprisingly, minimal functional differences in drought tolerance where detected among the three life ferns from pressure volume curve parameters (Figure 2a). The turgor loss point (tlp) varied by fern life form (*P* = 0.042), however, post-hoc multiple comparisons did not detect differences in tlp across terrestrial, hemi-epiphytic or epiphytic ferns. Broadly, terrestrial and hemi-epiphytic fern species had slightly lower tlp than epiphytic species (Figure 2b). Additionally, the osmotic potential (o) decreased in terrestrial and hemi-epiphytic species (*P* = 0.009, Figure 2C), while the modulus of elasticity () was similar across all life forms. Consequently, the broad decreases in tlp among terrestrial and hemi-epiphytic fern species appeared to be driven by osmotic adjustments via solute accumulations rather than shifts in cell wall flexibility (see Bartlett *et al.* (2012)).

Tissue capacitance at turgor loss point (**capacitance zero?**) was 54 % lower in epiphytic compared to terrestrial species (*P* = 0.010), with hemi-epiphytic species has intermediates. **add normal capactiance instead**

## Phylogentic traits relationships:

* density vs size (not much with stomata and other traits)

# Figures

 Figure 1. Structural shifts in path lengths to transport water, via stipe length, alters how tropical fern life forms build conductive structures. (A) Stipe length is reduced in epiphytic tropical fern species that develop without permanent connections to soil. (B) Lamina area in tropical ferns is allometrically (log-based) related to stipe length and this relationship varies by life form. The conditional and marginal r2 for the overall allometric relationship between lamina area and stipe length are 0.23 and 0.88, respectively. Significant log-linear fits for each life from are shown with dashed lines. The width of boxplots are drawn proportional to the number of observations in each life form.

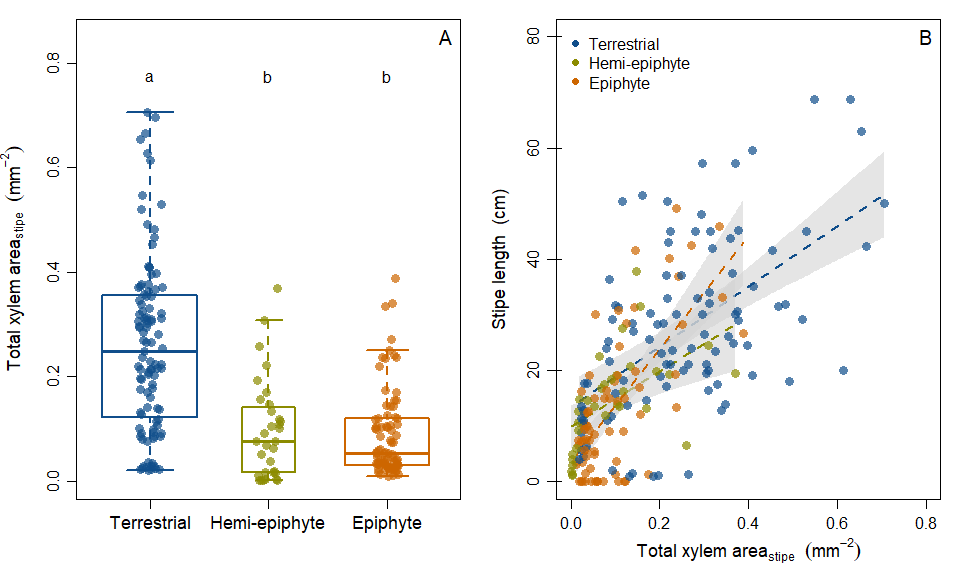
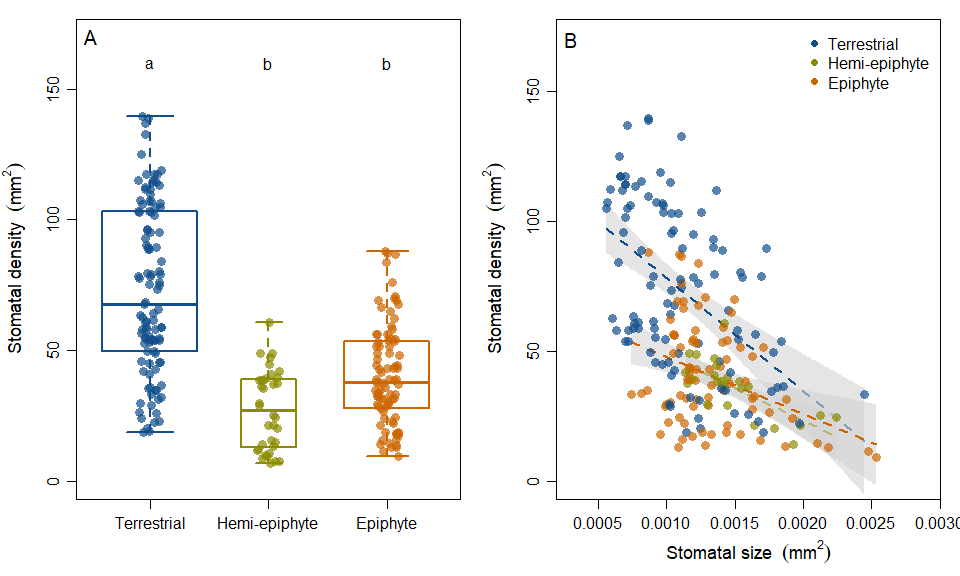
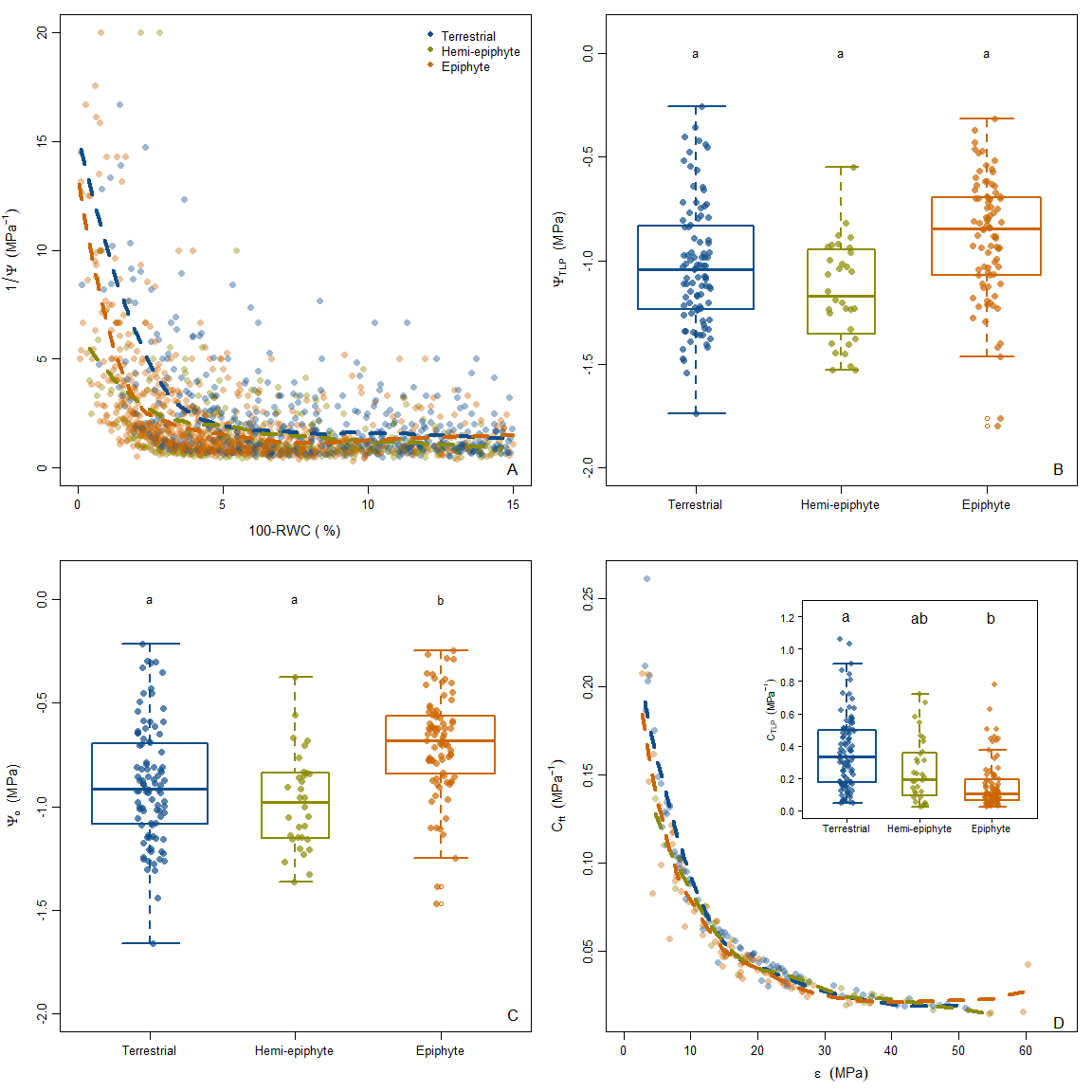
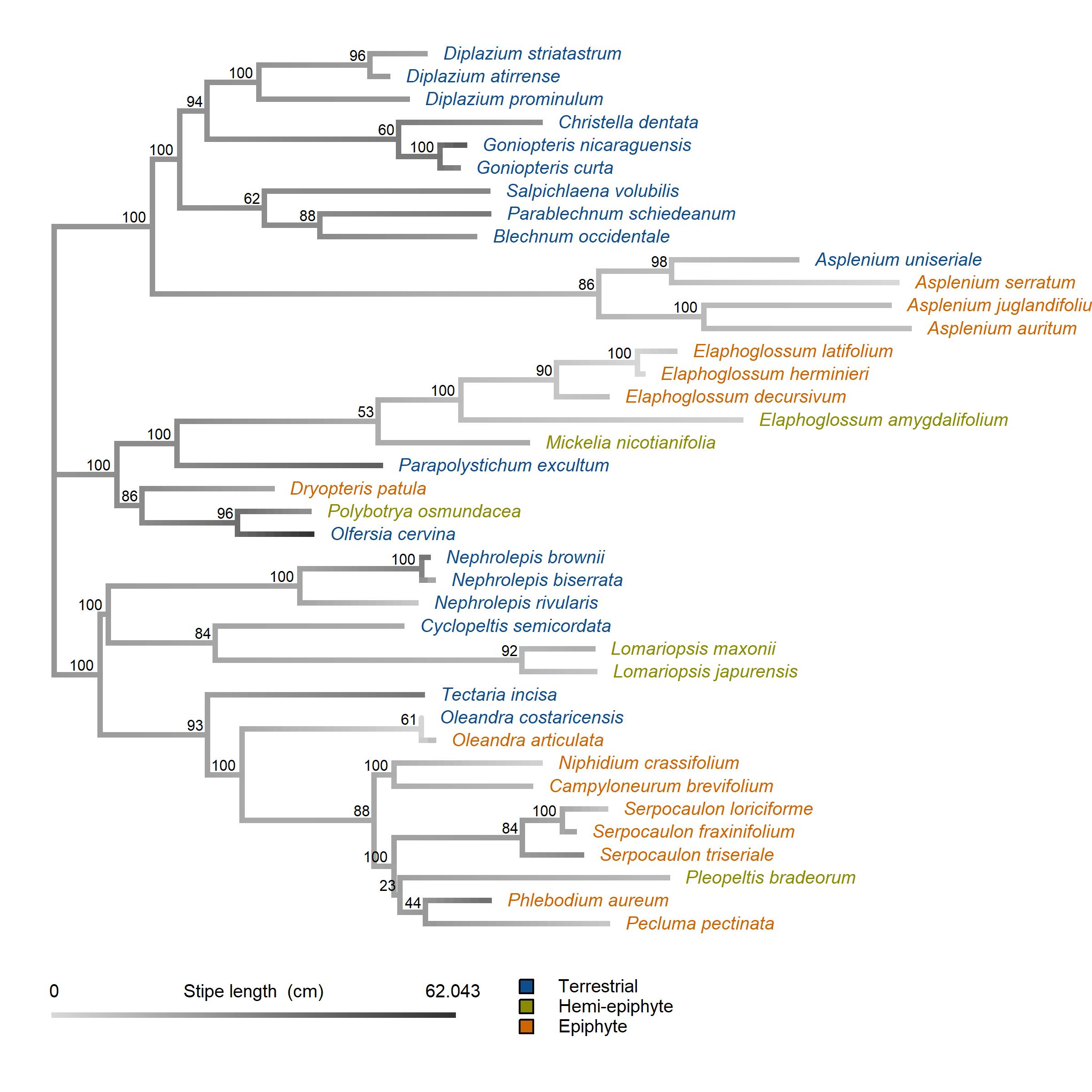
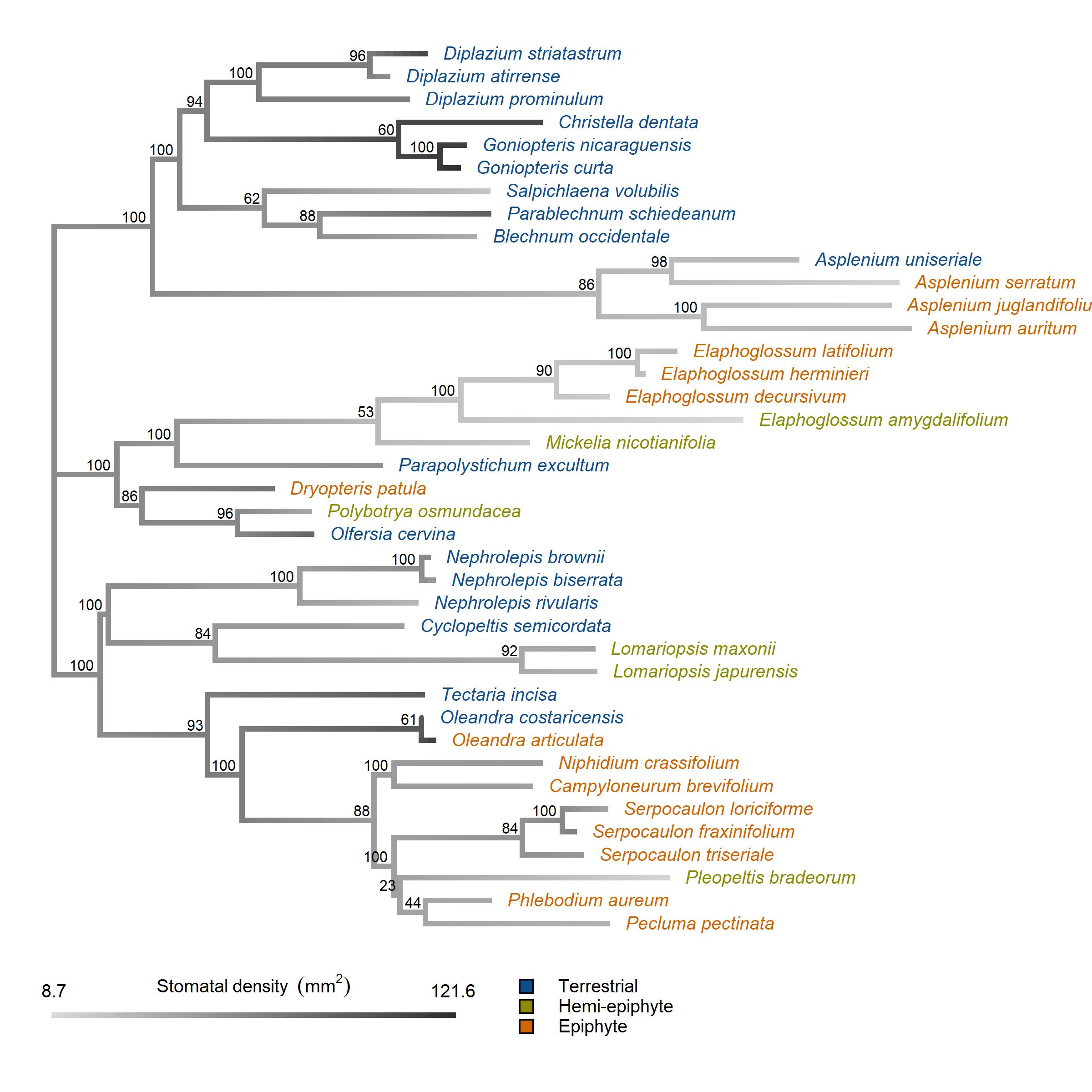
 Figure 2. Conductive hydraulic supply, via total xylem area, constrains stipe lenth in tropical fern species. (A) Total xylem area is both reduced and constained in tropical epiphitic and hemi-epiphytic ferns. (B) The capacity for greater total xylem area in terretrial tropical ferns supports construction of large stipes. The conditional and marginal r2 for the overall postive relationship between stipe length and total xylem area are 0.30 and 0.88, respectively. Significant linear relationships for each life from are shown with dashed lines representing model fits and grey shaded areas as 95 % confidence intervals for the mean.The width of boxplots are drawn proportional to the number of observations in each life form.

Figure 3. Shifts in lamina thickness (LMA) and lamina chemistry differ between tropical fern life forms. (A) Epiphytic ferns have greater LMA than non-epiphytic ferns and (B) LMA is negatively related to lamina nitrogen content in only terrestrial and hemi-epiphytic ferns. (C) Lamina 13C was higher in epiphytic ferns (higher values indicate higher water-use efficiency) compared to terrestrial and epiphytic ferns. Significant linear relationships are shown with dashed lines representing model fits and grey shaded areas as 95 % confidence intervals for the mean. The width of boxplots are drawn proportional to the number of observations in each life form.

 Figure 4. Stomatal traits differed between tropical fern life forms. (A) Stomatal density was reduced in epiphytic and hemi-epiphytic ferns compared to terrestrial ferns. (B) Stomatal density was negatively related to stomatal size for all life forms. Significant linear relationships for each life from are shown with dashed lines representing model fits and grey shaded areas as 95 % confidence intervals for the mean.The width of boxplots are drawn proportional to the number of observations in each life form.

 Figure 5. Epiphytic ferns species are not more drought tolerant. Osmotic adjustments, not shifts in cell wall flexibility explain differences in TLP. I am not sure what capacitance at TLP means.

  
Figure 6. Ancestral state reconstruction for stipe length mapped onto the phylogeny of the selected ferns examined in this study. A bootstrap value is associated with each node.

  
Figure 7. Ancestral state reconstruction for stomatal density mapped onto the phylogeny of the selected ferns examined in this study. A bootstrap value is associated with each node.

# Tables

Table 1. Phylogenetic signal for X functional traits of tropical ferns sampled across the Euploypoid I & II clades. Meausured species (n=39) encompass tererstrial, hemi-spiphytic and epiphytic habitats across two Costa Rican forest sites. **Eventually we will drop some of these**

|  |  |  |
| --- | --- | --- |
| **Functional Trait** | **K-statistic** | ***P*** |
| Stomatal Density | 0.5801065 | 0.001 |
| Stomatal Size | 0.3567476 | 0.024 |
| Guard cell length | 0.2940968 | 0.063 |
| Guard cell width | 0.31346 | 0.036 |
| Xylem area | 0.2720322 | 0.137 |
| Huber value | 0.033933 | 0.99 |
| TLP | 0.2682495 | 0.107 |
| Osmotic Potential | 0.316065 | 0.047 |
| Elasticity | 0.1459465 | 0.578 |
| Capacitance full | 0.1401441 | 0.65 |
| Capacitance zero | 0.1326652 | 0.678 |
| Frond length | 0.2219753 | 0.318 |
| Stipe length | 0.2546361 | 0.186 |
| Lamina area | 0.2865205 | 0.215 |
| Chlorophyll content | 0.189138 | 0.376 |
| SLA | 0.2107074 | 0.311 |
| 13C | 0.2179485 | 0.228 |
| Foliar Nitrogen | 0.3084941 | 0.037 |

# Discussion

## Do biomechanical relationships define water transport capacity?

* Differences in slopes of scaling realtionships between epiphyitc and terrestrial (more than with phyloegny) ferns have been detected with LMA and petiole width, suggesting a balance is required between optimizing hydaulic support and providing sufficient biomechanical support (Peppe *et al.*, 2014)
* Foliar Narea increases with LMA () in terrestrial temperate ferns understory (Karst & Lechowicz, 2007) add campany  
  **Broadly, you cannot have a large stipe without large xylem area**

## Do canalized traits define drought avoidance?

## where do hemi-epiphytes fit?

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We would like to thank Juliette Bechard, Kathleen Bynon, Luke Calderaro and Alexandra Russell for their hard work in the field and in the lab. We would also like to thank Rodolfo Quiros Flores and Bernal Matarrita Carranza for their organizational support at each OTS field station.

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