

Convergence of ecophysiological traits drives floristic composition of early lineage vascular plants in a tropical forest floor

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- **Background and Aims** Tropical understorey plant communities are highly diverse and characterized by variable resource availability, especially light. Plants in these competitive environments must carefully partition resources to ensure ecological and evolutionary success. One mechanism of effective resource partitioning is the optimization of functional traits to enhance competition in highly heterogeneous habitats. Here, we surveyed the ecophysiology of two early lineage vascular plant groups from a tropical forest understorey: *Selaginella* (a diverse lineage of lycophytes) and ferns.
- **Methods** In a lowland rain forest in Costa Rica, we measured a suite of functional traits from seven species of *Selaginella* and six fern species. We evaluated species microclimate and habitat; several photosynthetic parameters; carbon, nitrogen and phosphorus content; chlorophyll concentration; leaf mass per area (LMA); and stomatal size and density. We then compare these two plant lineages and search for relationships between key functional parameters that already exist on a global scale for angiosperms.
- **Key Results** Convergence of trait function filtered *Selaginella* species into different habitats, with species in heavily shaded environments having higher chlorophyll concentrations and lower light compensation points compared with open habitats. Alternatively, lower foliar nitrogen and higher stomatal densities were detected in species occupying these open habitats. *Selaginella* species had denser and smaller stomata, lower LMA and lower foliar nutrient content than ferns, revealing how these plant groups optimize ecophysiological function differently in tropical forest floors.
- **Conclusions** Our findings add key pieces of missing evidence to global explorations of trait patterns that define vascular plant form and function, which largely focus on seed plants. Broadly predictable functional trait relationships were detected across both *Selaginella* and ferns, similar to those of seed plants. However, evolutionary canalization of microphyll leaf development appears to have driven contrasting, yet successful, ecophysiological strategies for two coexisting lineages of extant homosporous vascular plants.

Key words: *Selaginella*, lycophytes, ferns, photosynthesis, microphyll, stomata, nitrogen, LMA

INTRODUCTION

The genus *Selaginella* is the largest extant lineage of lycophytes and consists of >700 species (PPG I, 2016; Weststrand and Korall, 2016; Zhou *et al.*, 2016). Most members of the genus produce simple dichotomously branched stems with diminutive microphyll-like leaves. This simple body plan has exhibited notable morphological stasis, and modern taxa are outwardly similar to the oldest known Carboniferous fossils (Thomas, 1992). This simplistic morphology belies remarkable ecological diversity, with species distributed across an array of habitats: from xeric desert-like conditions to swamps in ever-wet tropical rain forests and high-elevation alpine meadows (Korall and Kendrick, 2002; Arrigo *et al.*, 2013). Along with this ecological breadth comes a wide range of physiological function as the genus consists of some of the most desiccation-tolerant and drought-sensitive vascular plants on earth (Black and Pritchard, 2002). In addition to such functional diversity, *Selaginella* is an unusual lineage that is sister to the largely aquatic–amphibious genus *Isoetes* both of which represent one of the earliest evolutionary experiments with heterospory in vascular plants.

Much attention has been paid to the evolution of the lycophytes (e.g. Thomas, 1992). Yet, it is only relatively recently that botanists have begun to understand the phylogeny of *Selaginella*. A series of elegant studies have helped develop a better picture of species relationships in a diverse sets of species (Korall and Kendrick, 2002; Arrigo *et al.*, 2013; Zhou *et al.*, 2016; Weststrand and Korall, 2016). In spite of this, we know surprisingly little about the ecology of the genus. Work done on lycophytes suggests that some species can maintain unexpectedly high maximum photosynthetic rates and produce long-lived leaves (Brodrribb *et al.*, 2007). A great deal of recent interest has focused on stomatal function, with studies suggesting novel ways that lycophyte and fern stomata may function relative to seed plants (Franks and Farquhar, 2007; Brodrribb *et al.*, 2009; Brodrribb and McAdam, 2011; Ruszala *et al.*, 2011; McAdam and Brodrribb, 2012a, 2013). Yet, outside of detailed studies of desiccation tolerance, research examining the *in situ* ecophysiology of *Selaginella* is very limited. This lack of knowledge inhibits our ability to compare the ecophysiology of this group with that of other plant lineages.

While ferns are more closely related to seed plants, ferns and *Selaginella* share unique ecological and reproductive

characteristics that set them apart from seed plants (Brodribb and Holbrook, 2004; Moran, 2004). Both groups produce spores, independent gametophytes and motile swimming sperm. While both groups are widely distributed into xeric conditions, ecologically ferns and *Selaginella* often share similar damp low-light habitats. An important distinction between the two groups is the leaf: *Selaginella* represents an evolutionarily successful experiment that relies on microphyll-like leaves (often referred to as lycophylls) while ferns produce large megaphylls.

The lycophytes first appeared in the Late Silurian as one of the earliest sporophyte-dominated lineages of vascular plants, with stomata specifically designed for CO₂ and water regulation (Edwards, 1993; Edwards et al., 1998; Brodribb and Holbrook, 2004), a characteristic that may have allowed for the functional diversification of land plants we see today. Ferns appeared later during the Mid-Devonian (Schneider et al. 2004; Sharpe et al., 2010) during a time when CO₂ levels were 2200 ppm or eight times pre-industrial levels (Bernier and Kothavala, 2001), compared with the nearly 16 times pre-industrial level at 4500 ppm during the Late Silurian. Even though most ferns and lycophyte species are no older than seed plants, the former carry with them a vestige of evolutionary canalization from their ancestors, yet we know little of their modern ecophysiological function.

Here we report novel data investigating physiological, anatomical, and stoichiometric leaf-level parameters for lycophytes and ferns in a wet tropical rain forest. We focus on key functional parameters that encompass physiological behaviour, resource investment and life history strategies in the understudied genus *Selaginella* and compare this with ferns growing in similar habitats. We sought to understand the functional ecology that allows ferns and lycophytes to coexist in tropical forest floors. Importantly, the distribution of *Selaginella* species surveyed in this study overlaps entirely with the chosen terrestrial fern species. Relationships between these functional parameters are already shown to exist on a global scale for angiosperms (see Wright et al., 2004), but are poorly represented in these important early lineages of land plants.

MATERIALS AND METHODS

Site description

This study was located at La Selva Biological Research Station in Heredia, Costa Rica (84°00'12W, 10°25'52N) and took place in June of 2011. This site is a 600 ha wet tropical forest, with elevation ranging from 35 to 137 m above sea level. The site receives 4 m of annual rainfall with a moderate dry season. Annual mean temperatures fluctuates between 24 and 27 °C, while daily temperatures can fluctuate up to 12 °C (McDade et al., 1994).

Species selection and habitat microclimate

A survey of physiological and stoichiometric parameters was conducted for seven terrestrial *Selaginella* and six terrestrial fern species (Table 1). Vouchers for *Selaginella* were deposited at the La Selva Herbarium (LSCR) with duplicates deposited at George R. Cooley Herbarium at Colgate University (GRCH). All measurements and sample collections were conducted

in situ during mid-morning. At the study site, species in the study were found across distinct terrestrial niches, characterized by different light environments, temperature and humidity. Specifically, surveys were conducted in (1) open-canopy areas alongside riverbeds, (2) closed-canopy swamps and (3) closed-canopy forest floors. These classifications were reached based on personal observations (J. E. Watkins) and results from Watkins et al. (2007, 2010) and Watkins and Cardelús (2009), and represent a large proportion of the microclimatic variation of tropical forest floor where the surveyed species are established. To quantify microclimate parameters, a HOBO data logger (Onset Computer Corporation, Bourne, MA, USA) was placed in representative areas of each of the three habitats to record air temperature (°C), relative humidity and dew point temperature (°C). Data loggers were run simultaneously and were left in place for 36 h, and microclimate parameters were recorded at 10 min intervals.

Photosynthetic parameters

For leaf gas exchange measurements, a LiCor 6400 portable photosynthesis system (LiCor BioSciences, Lincoln, NE, USA) was used with the standard leaf chamber (2 × 3 cm) equipped with blue–red light-emitting diodes. The CO₂ mixer was set to 400 μmol m⁻² s⁻¹ and no explicit temperature set point was used. Prevailing temperatures for measurements ranged from 27 to 31 °C. Gas exchange parameters were logged once CO₂ and water vapour fluxes were stable. Net photosynthetic rate (A_n) and stomatal conductance (g_s) were logged at a standard photosynthetic photon flux density (PPFD) level of 500 μmol m⁻² s⁻¹, which has been previously shown to produce maximum photosynthetic rates for shade-adapted understory terrestrial plants (Watkins et al., 2010). These forest floor-dwelling plants were measured during morning hours to obtain maximum values before plants shut down in the mid-day heat (Karst and Lechowicz, 2006). One fully developed pinna in the case of ferns or a section of above-ground tissue in *Selaginella* was measured for gas exchange on each of five individuals per species. Leaf-level instantaneous water use efficiency (WUE) was calculated as A_n divided by transpiration from gas exchange measurements.

Light response curves were conducted by decreasing the light intensity in the gas exchange cuvette in small steps, resulting in a 13 step change response curve occurring over approx. 45 min. Leaves were acclimated at 1000 PPFD until CO₂ and water vapour fluxes were stable. Next, we proceeded with 180 s step decreases in irradiance intensity: 1000, 750, 500, 300, 250, 150, 100, 50, 35, 25, 10, 5 and 0 PPFD. The light compensation point (LCP) was calculated as the PPFD at which the net photosynthetic rate equalled zero from the linear phase of each light response curve.

Stoichiometry

Foliar tissue was collected following gas exchange measurements and used for nutrient analyses. Samples were dried to a constant mass and ground using a Wig-L-Bug (Sigma-Aldrich Co. St. Louis, MO, USA). Carbon and nitrogen analyses were measured

TABLE 1. *List of selected species of genus Selaginella and ferns surveyed at La Selva Biological Field Station*

Species	Family	Habitat	Abbreviation
<i>Selaginella arthritica</i>	Selaginellaceae	Closed canopy	Sel_art
<i>Selaginella atirrensis</i>	Selaginellaceae	Closed canopy	Sel_ati
<i>Selaginella eurynota</i>	Selaginellaceae	Closed canopy	Sel_eur
<i>Selaginella</i> sp.	Selaginellaceae	Open canopy	Sel_hue
<i>Selaginella umbrosa</i>	Selaginellaceae	Open canopy	Sel_umb
<i>Selaginella anceps</i>	Selaginellaceae	Swamp	Sel_anc
<i>Selaginella oaxacana</i>	Selaginellaceae	Swamp	Sel_oax
<i>Bolbitis portoricensis</i>	Dryopteridaceae	Closed canopy	Bol_por
<i>Cyclopetlis semicordata</i>	Lomariopsidaceae	Closed canopy	Cyc_sem
<i>Diplazium striatastrum</i>	Athyriaceae	Closed canopy	Dip_str
<i>Lomariopsis japurensis</i>	Lomariopsidaceae	Closed canopy	Lom_jap
<i>Thelypteris curta</i>	Thelypteridaceae	Closed canopy	The_cur

All surveyed species occupy terrestrial (non-epiphytic) habitats. Individual species abbreviations are provided for reference in the figures. *Selaginella* sp. is likely in the *S. huehuetenangensis* group, but identification remains unclear.

using a Costech Analytical Elemental Analyzer (Valencia, CA, USA), with the percentage of carbon and nitrogen in samples calculated by comparison with certified standards. Foliar phosphorus concentrations were determined using an ash digestion process (D'Angelo *et al.*, 2001) preceded by colour development and absorbance measurement on an Astoria Pacific colorimetric autoanalyser (Clackamas, OR, USA). Photosynthetic nitrogen use efficiency (PNUE) was defined as the ratio of photosynthesis to leaf nitrogen content on a mass basis.

Chlorophyll content

For each *Selaginella* species, sub-samples of fresh foliar tissue used during gas exchange measurements were used to calculate chlorophyll content (Chl). Chlorophyll was extracted using dimethylformamide, and absorbances were measured in a spectrophotometer at 647 and 664.5 nm (Jacobsen *et al.*, 2012). Concentrations of chlorophyll *a*, *b* and total chlorophyll (mg L⁻¹) were calculated using the equations given by Inskeep and Bloom (1985). Here, chlorophyll *a*, *b* and total chlorophyll are reported on a mass basis (mg Chl g fresh leaf mass⁻¹)

Leaf anatomy

Stomatal density (SD) was measured by directly counting stomata on the abaxial leaf surface under ×40 magnification with a field of view of 0.46 mm². Stomatal density for each field of view was calculated from ten non-overlapping regions on five individuals of each fern and *Selaginella* species. Individual stomata size, calculated as guard cell length (mm), was also measured on ten total stomata for each individual foliar sample.

Leaf mass per unit area (LMA) was measured for both lycophyte and fern species from the same individuals used for leaf gas exchange. For fern species, LMA was calculated using the tissue punch method (1 mm², *n* = 10) as dry mass divided by area. Due to overlapping and small microphylls, characteristic of *Selaginella* species, ten leaf samples for each individual of *Selaginella* were excised, photographed to scale and analysed for area using ImageJ (NIH, Bethesda, MD, USA). Excised

samples included small sections of green stem tissue. All samples were then dried to a constant mass to calculate LMA.

Data analysis

Principal component analysis, utilizing the ‘vegan’ package (Oksanen *et al.*, 2018), was used to explore how measured functional traits were distributed and co-varied among *Selaginella* taxa. Tukey’s post-hoc test were performed in conjunction with analysis of variance (ANOVA) to determine which mean values of functional traits were different among *Selaginella* taxa with the ‘multcomp’ package (Hothorn *et al.*, 2008). Functional trait patterns that were statistically driven by a single species are not discussed. For bivariate trait relationships, responses of dependent variables were analysed with linear mixed-effect models, with species as a random effect and habitat (*Selaginella* only) as a categorical fixed effect. Differences in slopes of the relationship of bivariate traits by habitat (*Selaginella* only) were tested by calculating estimated marginal means and computing pairwise comparisons with the ‘emmeans’ package (Length, 2018). Explained variance (*R*²) of mixed models was computed as in Nakagawa and Schielzeth (2013), in which the marginal *R*² represents variance explained by fixed factors and the conditional *R*² represents variance explained by both fixed and random factors. *T*-tests were performed to test for differences between pooled functional traits of *Selaginella* and fern species. All tests of statistical significance were conducted at an α level of 0.05. All analyses were performed with R 3.4.3 (R Development Core Team, 2017).

RESULTS

Habitat microclimate

In addition to the prevailing light environment, each habitat zone was characterized by different driving environmental variables. Diurnal fluctuations in temperature and vapour pressure deficit (VPD) varied across each site (Fig. 1). For example, during the diurnal period displayed in Fig. 1, maximum air temperature varied from 27.6 to 29.1 °C and maximum VPD varied from 0.07 to 0.42 kPa across habitat zones. Overall, non-overlapping

compositions of *Selaginella* taxa occurred in the forest floor of each of these distinct habitats at this tropical site (Table 1).

Ecophysiology of different Selaginella taxa

We first explored how each of 11 measured functional traits varied and were distributed among *Selaginella* taxa. In total, 60.1 % of the trait variation was accounted for in two dimensions, encompassing different resource economics, physiology and life strategies of the studied *Selaginella* taxa. Interestingly, there

was minimal overlap among component scores for many of the surveyed species, and species associations with functional traits were noticeably divergent (Fig. 2). These disparate relationships with measured functional traits suggest a highly variable *in situ* ecophysiology for coexisting *Selaginella* taxa. Indeed, a large amount of variation was detected across *Selaginella* species with measured variables (Tables 2 and 3). Overall, differences between habitat types were apparent in leaf stoichiometry and functional traits, but not in leaf-level physiology.

Rates of net photosynthesis differed across surveyed *Selaginella* species ($P < 0.001$, $F = 23.7$) and varied by up to 70

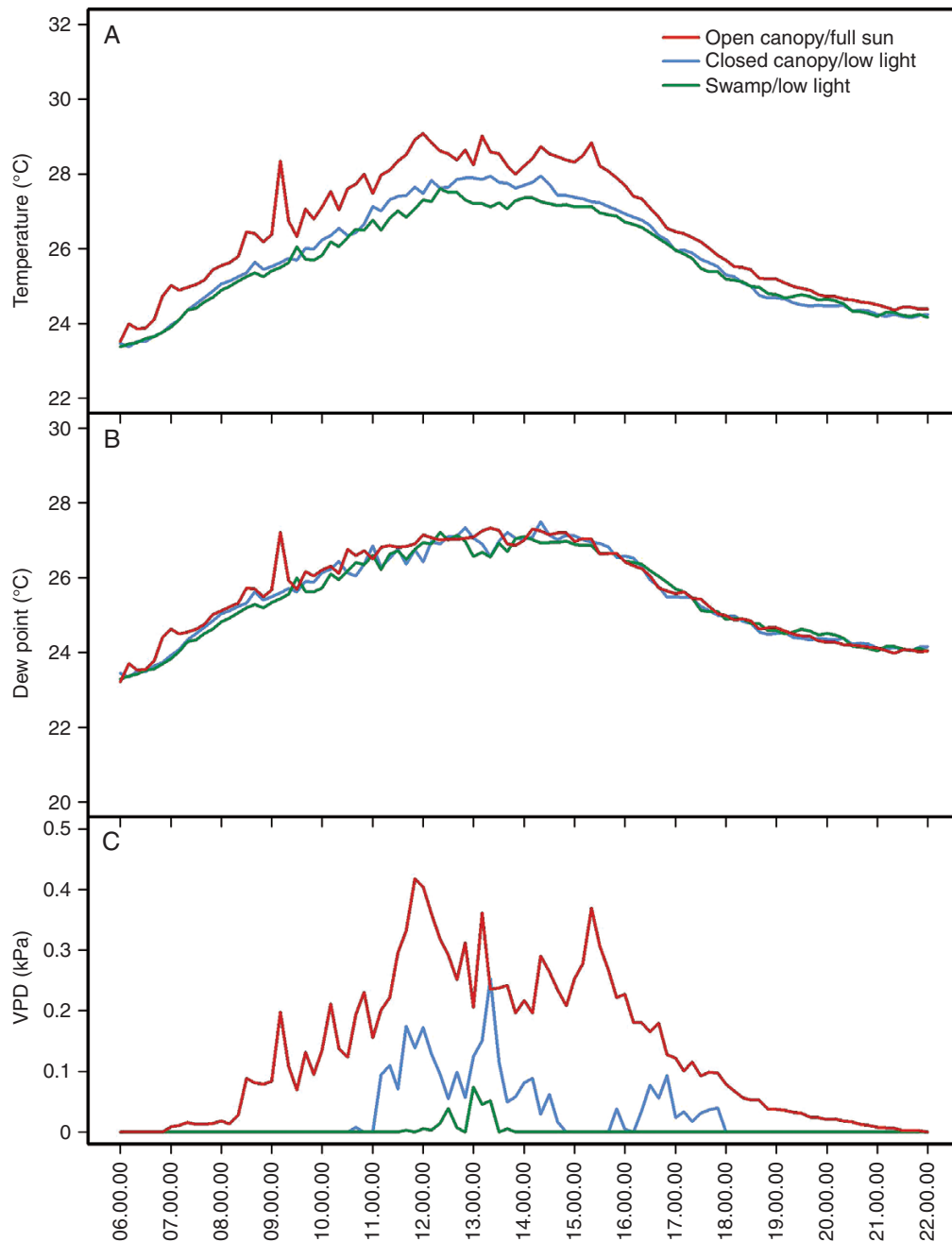


FIG. 1. Environmental variables measured over a typical diurnal period: air temperature (A), dew point temperature (B) and vapour pressure deficit (C). *Selaginella* species were established and measurements were recorded in each habitat zone.

%). Stomatal conductance differed between *Selaginella* species ($P < 0.001$, $F = 8.56$) and varied by up to 58 %. Instantaneous WUE differed between *Selaginella* species ($P = 0.004$, $F = 4.13$) and varied by up to 38 %. No effect of habitat type was detected on either A_n , g_s or WUE.

Total leaf chlorophyll content differed among *Selaginella* species ($P = 0.010$, $F = 3.57$) and by habitat type ($P = 0.005$, $F = 6.12$). Total leaf chlorophyll content varied by up to 37 % between species and was higher in species occupying low-light swamps compared with open habitats. Chlorophyll *a* differed among *Selaginella* species ($P = 0.002$, $F = 4.68$) and by habitat ($P = 0.010$, $F = 5.37$). Chlorophyll *b* also differed among *Selaginella* species ($P = 0.014$, $F = 3.28$) and by habitat ($P = 0.020$, $F = 4.45$). Both chlorophyll *a* and *b* were higher in species occupying low-light swamps than open habitats. The ratio of chlorophyll *a* and *b* varied among *Selaginella* species ($P < 0.001$, $F = 8.08$) but was similar across habitat types. The LCP varied among *Selaginella* species ($P < 0.001$, $F = 20.05$) and habitat types ($P < 0.001$, $F = 9.64$). Across species, the amount of light where net CO₂ assimilation was zero varied from 0.7 to 10.2 PPFD. Overall, LCP was lower in the low-light swamp compared with open habitats.

Leaf N differed among *Selaginella* species ($P < 0.001$, $F = 17.13$) and between habitat types ($P < 0.001$, $F = 27.67$). Leaf N content varied by up to 57 % and was lowest in species occupying open habitats. A similar pattern was also detected in leaf C:N, which varied between species ($P < 0.001$, $F = 12.65$) and was highest in open habitats ($P < 0.001$, $F = 38.35$). Leaf phosphorus also differed among *Selaginella* species ($P < 0.001$, $F = 13.49$) and between habitat types ($P < 0.001$, $F = 6.50$). Leaf phosphorus content varied by up to 70 % and was higher in the closed-canopy forest floor habitat (although probably driven by only *Selaginella eurynota*).

The LMA differed among *Selaginella* species ($P < 0.001$, $F = 26.85$) and habitat type ($P = 0.021$, $F = 4.36$). Lower LMA in *Selaginella atirrensis* largely drove the habitat response, leading to lower LMA in the closed-canopy forest floor habitat. Stomatal density varied widely between *Selaginella* species ($P < 0.001$, $F = 58.71$) and habitat type ($P < 0.001$, $F = 62.91$). Stomatal densities ranged from 26 to 117 (counts mm⁻²) and were the highest in species occupying open habitats (Fig. 3A). Stomatal size, via guard cell length, also differed among *Selaginella* species ($P < 0.001$, $F = 74.65$) and habitat types ($P < 0.001$, $F = 10.85$). Stomatal

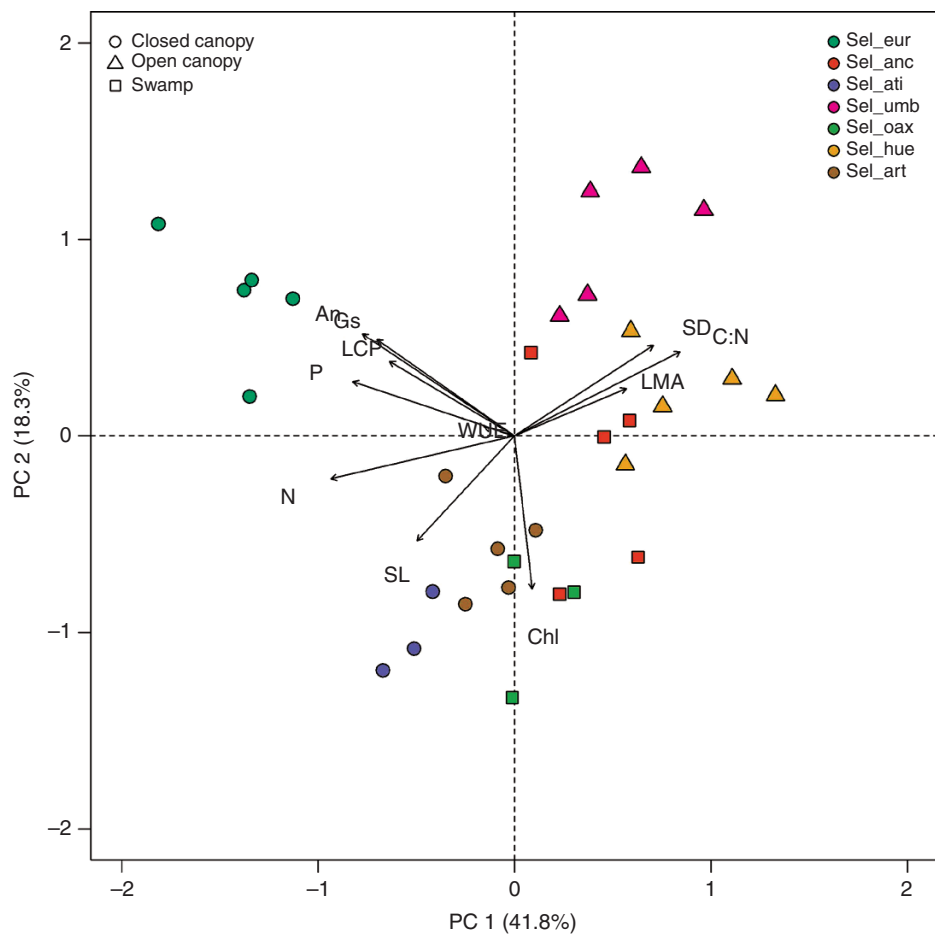


FIG. 2. Redundancy analysis of measured functional traits across *Selaginella* species in a wet tropical forest floor. With the 11 measured variables, 60.1 % of the trait variation was accounted for in two dimensions. Variables include net photosynthesis (A_n), stomatal conductance (g_s), instantaneous water use efficiency (WUE), total chlorophyll content (Chl), stomatal length (SL), stomatal density (SD), leaf mass per unit area (LMA), the light compensation point (LCP), foliar nitrogen (N) and phosphorus (P) content, and the leaf carbon to nitrogen ratio (C:N).

TABLE 2. Leaf-level physiological parameters of terrestrial *Selaginella* species surveyed in a wet tropical forest

Species	A_n ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	g_s ($\text{mol m}^{-2} \text{s}^{-1}$)	WUE ($\mu\text{mol CO}_2 \text{ mmol H}_2\text{O}^{-1}$)	Chlorophyll <i>a</i> (mg g per leaf)	Chlorophyll <i>b</i> (mg g per leaf)	LCP (PPFD)
<i>S. anceps</i>	3.1 (0.10) ^{bc}	0.07 (0.002) ^{ab}	4.1 (0.26) ^{ab}	6.4 (0.39) ^{ab}	4.4 (0.41) ^a	1.68 (0.777) ^a
<i>S. arthritica</i>	3.4 (0.23) ^c	0.08 (0.005) ^{ab}	4.6 (0.46) ^b	6.8 (0.42) ^{ab}	3.1 (0.32) ^a	2.38 (0.438) ^a
<i>S. atirrensis</i>	1.8 (0.25) ^a	0.06 (0.008) ^a	3.0 (0.389) ^a	6.0 (0.40) ^{ab}	4.4 (0.30) ^a	8.16 (1.091) ^{cd}
<i>S. eurynota</i>	5.8 (0.49) ^d	0.12 (0.013) ^c	4.7 (0.09) ^b	4.5 (0.38) ^a	2.9 (0.14) ^a	10.17 (1.150) ^d
<i>S. oaxacana</i>	2.4 (0.13) ^{ac}	0.06 (0.006) ^{ab}	4.8 (0.30) ^b	7.6 (0.42) ^b	4.1 (0.32) ^a	0.66 (0.341) ^a
<i>S. species</i>	2.1 (0.28) ^{ab}	0.05 (0.007) ^a	4.0 (0.23) ^{ab}	5.5 (0.18) ^{ab}	3.0 (0.10) ^a	5.88 (0.508) ^{bc}
<i>S. umbrosa</i>	3.5 (0.28) ^c	0.10 (0.011) ^{bc}	4.2 (0.27) ^{ab}	4.7 (0.33) ^a	3.0 (0.28) ^a	3.36 (0.374) ^{ab}
<i>P</i> -value	0.152	0.004	0.281			

Values for net photosynthesis (A_n), stomatal conductance (g_s), instantaneous water use efficiency (WUE), chlorophyll *a* and *b* content and the light compensation point (LCP) represent the mean (± 1 s.e.) of individually measured plants ($n = 5$).

Letters represent significant differences between species. Different letters represent significant differences between species.

The *P*-value represents overall differences between *Selaginella* and fern species, when trait comparisons were available.

TABLE 3. Leaf-level anatomy and stoichiometry of terrestrial *Selaginella* species surveyed in a wet tropical forest

Species	LMA (g m ⁻²)	Leaf N (%)	Leaf C:N	Leaf P (%)	Stomatal length (μm)	Stomatal density (mm ⁻²)
<i>S. anceps</i>	12.8 (0.83) ^c	2.3 (0.16) ^{bc}	17.5 (1.25) ^a	0.16 (0.024) ^c	18.7 (0.57) ^a	65.4 (3.30) ^{de}
<i>S. arthritica</i>	12.5 (0.48) ^{de}	2.5 (0.09) ^c	15.0 (0.46) ^a	0.17 (0.026) ^{de}	26.1 (0.37) ^c	32.7 (2.32) ^{ab}
<i>S. atirrensis</i>	4.4 (0.47) ^a	2.8 (0.17) ^{cd}	14.2 (1.31) ^a	0.24 (0.020) ^a	26.0 (0.26) ^c	26.0 (1.82) ^a
<i>S. eurynota</i>	7.1 (0.44) ^b	3.5 (0.27) ^d	11.8 (0.77) ^a	0.37 (0.035) ^b	24.7 (0.44) ^{bc}	45.7 (3.39) ^{bc}
<i>S. oaxacana</i>	8.9 (0.70) ^{bc}	2.5 (0.15) ^c	15.8 (0.92) ^a	0.19 (0.023) ^{bc}	25.7 (0.74) ^c	60.0 (3.67) ^{cd}
<i>S. sp</i>	9.9 (0.45) ^{cd}	1.5 (0.15) ^a	26.7 (2.84) ^b	0.11 (0.009) ^{cd}	23.5 (0.42) ^b	117.8 (7.20) ^f
<i>S. umbrosa</i>	12.0 (0.72) ^{de}	1.6 (0.15) ^{ab}	26.2 (2.47) ^b	0.19 (0.116) ^{de}	17.0 (0.15) ^a	82.6 (4.72) ^e
<i>P</i> -value	0.001	0.001	0.001	0.001	0.001	0.001

Each values represents the mean (± 1 s.e.) of individually measured plants ($n = 5$). Different letters represent significant differences between species.

The *P*-value represents overall differences between *Selaginella* and fern species.

size was smaller in open canopy and swamp habitats, largely driven by *Selaginella umbrosa* and *Selaginella anceps* (Fig. 3C).

Selaginella vs. fern functional ecology

Neither A_n nor WUE was different between fern and *Selaginella* species; however, g_s was 13 % higher in ferns compared with *Selaginella* ($P = 0.004$). Foliar N content was 34 % higher in ferns compared with *Selaginella* species ($P < 0.001$), leading to lower C:N in fern taxa ($P < 0.001$). Foliar P content was also 31 % greater in ferns compared with *Selaginella* (both $P < 0.001$). The LMA was 50 % higher in ferns compared with *Selaginella* ($P < 0.001$). Importantly, there was negligible overlap in LMA between *Selaginella* and fern taxa. Generally, the surveyed *Selaginella* species had more dense and smaller stomata (both $P < 0.001$) than the surveyed fern species (Fig. 3B, D). All data for fern species used for comparison with *Selaginella* are given in Table 4.

Bivariate relationships between functional traits

Trait relationships with gas exchange parameters for both lineages broadly correspond to those predicted for seed plants. Across *Selaginella* species, A_n and g_s were positively

correlated ($P < 0.001$; Fig. 4A), and positive slopes of the relationship between A_n and g_s were similar among habitat types. A positive relationship between A_n and g_s was also detected in fern species, albeit less strongly ($P = 0.021$; Fig. 4B). Leaf-level photosynthesis was positively correlated to N content on a mass basis (N_m) across *Selaginella* species ($P = 0.009$; Fig. 4C) and positive slopes of the relationship between A_n and N_m were similar among habitat types. Leaf-level photosynthesis and N_m were not correlated for ferns (Fig. 4D), largely due to species differences. Leaf-level photosynthesis was also not related to phosphorus content for either *Selaginella* or ferns. Measured leaf gas exchange parameters were not related to stomatal traits across *Selaginella* or ferns species. Neither A_n nor g_s was correlated with either stomatal length or stomatal density for either plant lineage.

Photosynthesis on a mass basis (A_{mass}) was negatively correlated with LMA ($P = 0.003$) across *Selaginella* taxa, and slopes of this relationship were similar across habitats. The negative relationship between A_{mass} and LMA was also evident across fern species ($P < 0.001$). A decline in PNUE with increasing LMA was detected across fern species ($P < 0.001$) and *Selaginella* species ($P = 0.023$), regardless of habitat. Overall, PNUE was negatively correlated with LMA across all *Selaginella* and fern species ($P < 0.001$; Fig. 5). Leaf WUE was not related to PNUE for either *Selaginella* or fern taxa.

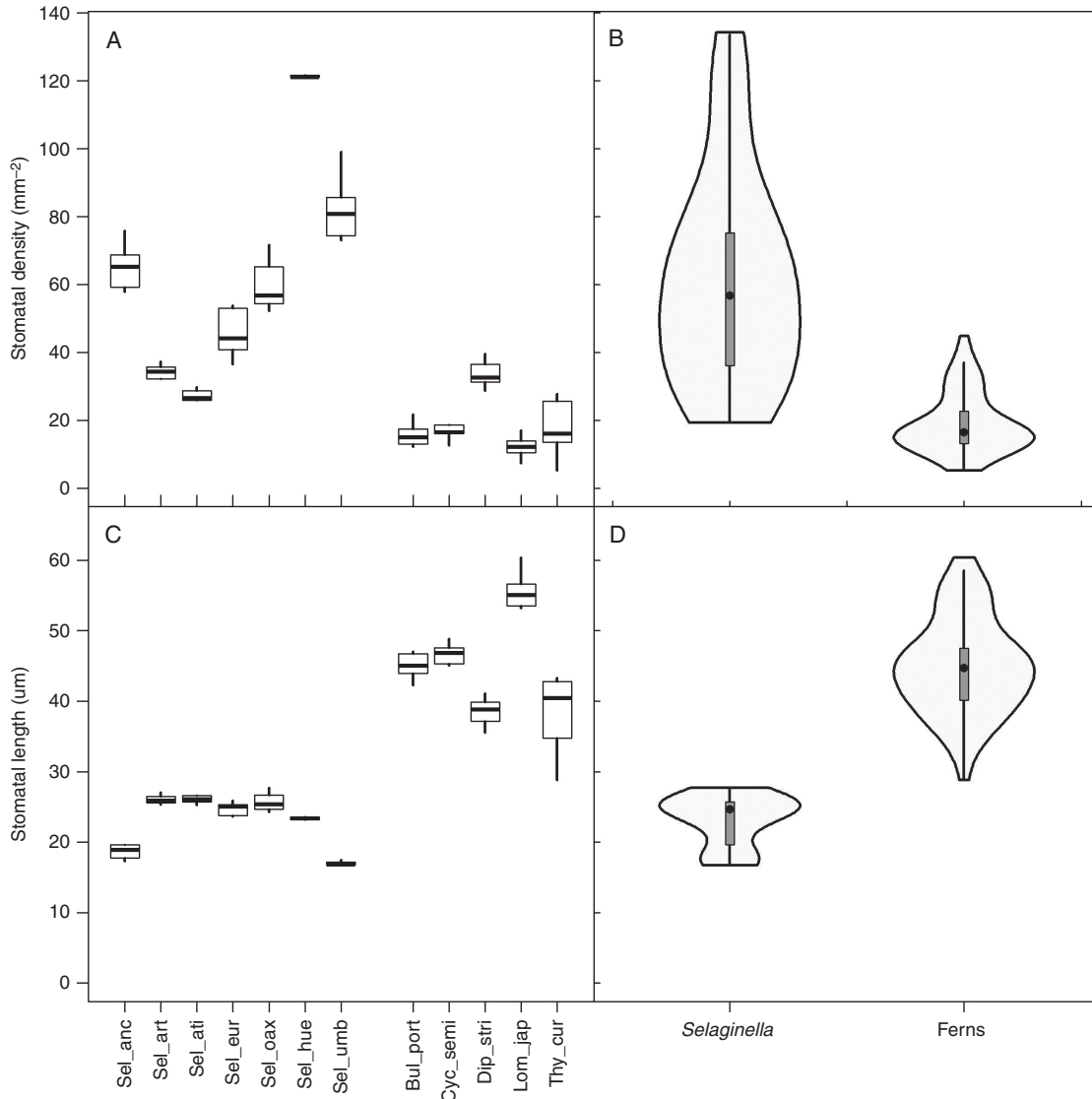


FIG. 3. Leaf stomatal traits differ strongly across *Selaginella* and fern taxa surveyed in a wet tropical forest. Stomatal density (A) and length (C) vary across species. Overall, violin plots show greater stomatal density (B) and smaller overall stomatal size (D) for *Selaginella* compared with ferns, with minimal overlapping kernel probability densities.

DISCUSSION

Vascular plants exist on a continuum of key trait patterns related to plant size, leaf economics and seed size (Diaz *et al.*, 2016). Some early lineage vascular plant taxa are conspicuously absent from these worldwide trait investigations, probably because they are small statured, difficult to work with, and few taxonomists are trained in identification of these groups. Although functional aspects of these early plant lineages underpin angiosperm evolution, the ecophysiology of extant taxa remains poorly quantified. Here, we investigate the *in situ* ecophysiology of understudied *Selaginella* taxa across distinct forest floor habitats and then compare patterns in functional traits with co-occurring terrestrial ferns. Our goal was to understand how both plant groups optimize physiological function in tropical forest floors. These findings offer new insights into how microphyll leaf development underpins differences in ecophysiological strategies between lycophytes and ferns.

Trait functional convergence of *Selaginella* taxa

Selaginella species have been shown to exhibit high family importance values in tropical forest understoreys (Lu *et al.*, 2011). Consequently, species distribution of *Selaginella* taxa probably contributes to tropical understorey structure and function. To begin to understand the role of lycophytes in forest floor communities, we must first build a foundation of the most basic aspects of their ecophysiology. Here, we focus on functional trait patterns related to leaf structure and physiology, one of the key trait strategy dimensions regulating how plants make a living (Wright *et al.*, 2007).

Few studies have explored *in situ* leaf-level gas exchange of extant lycopsids (see Zier *et al.*, 2015), and none in the tropics. We found a >2-fold range in A_n across seven tropical *Selaginella* species in their natural habitats. Measured rates of A_n fit within previously reported maximum photosynthetic rates (1.6–6.15 $\mu\text{mol m}^{-2} \text{s}^{-1}$) of two *Selaginella* species

TABLE 4. Leaf-level gas exchange, anatomy and stoichiometry of terrestrial fern species surveyed in co-occurring habitats as measured *Selaginella* species

Species	A_n ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	g_s ($\text{mol m}^{-2} \text{s}^{-1}$)	WUE ($\mu\text{mol CO}_2$ mmol H_2O^{-1})	LMA (g m^{-2})	Leaf N (%)	Leaf C:N	Leaf P (%)	Stomatal length (μm)	Stomatal density (mm^{-2})
<i>Bolbitis portoricensis</i>	3.2 (0.22)	0.06 (0.007)	5.9 (0.42)	14.9 (0.70)	4.2 (0.22)	10.3 (0.57)	0.32 (0.069)	45.0 (0.55)	15.8 (1.03)
<i>Cyclopetis semicordata</i>	2.2 (0.01)	0.07 (0.001)	4.2 (0.37)	21.8 (0.24)	3.0 (0.19)	14.3 (0.96)	0.32 (0.036)	46.7 (0.40)	17.0 (0.92)
<i>Diplazium striatastrum</i>	4.2 (0.32)	0.20 (0.011)	3.3 (0.25)	19.0 (0.30)	4.0 (0.07)	10.1 (0.25)	0.29 (0.031)	38.6 (0.56)	33.2 (2.11)
<i>Lomariopsis japurensis</i>	3.3 (0.35)	0.07 (0.169)	5.9 (0.13)	22.2 (0.27)	3.5 (0.36)	12.8 (1.43)	0.27 (0.028)	55.4 (0.69)	12.1 (0.83)
<i>Thelypteris curta</i>	3.8 (0.15)	0.18 (0.122)	3.1 (0.19)	18.2 (0.34)	3.3 (0.13)	11.7 (0.37)	0.27 (0.030)	38.9 (1.50)	17.0 (2.45)

Each value represents the mean (± 1 s.e.) of individually measured plants ($n = 5$).

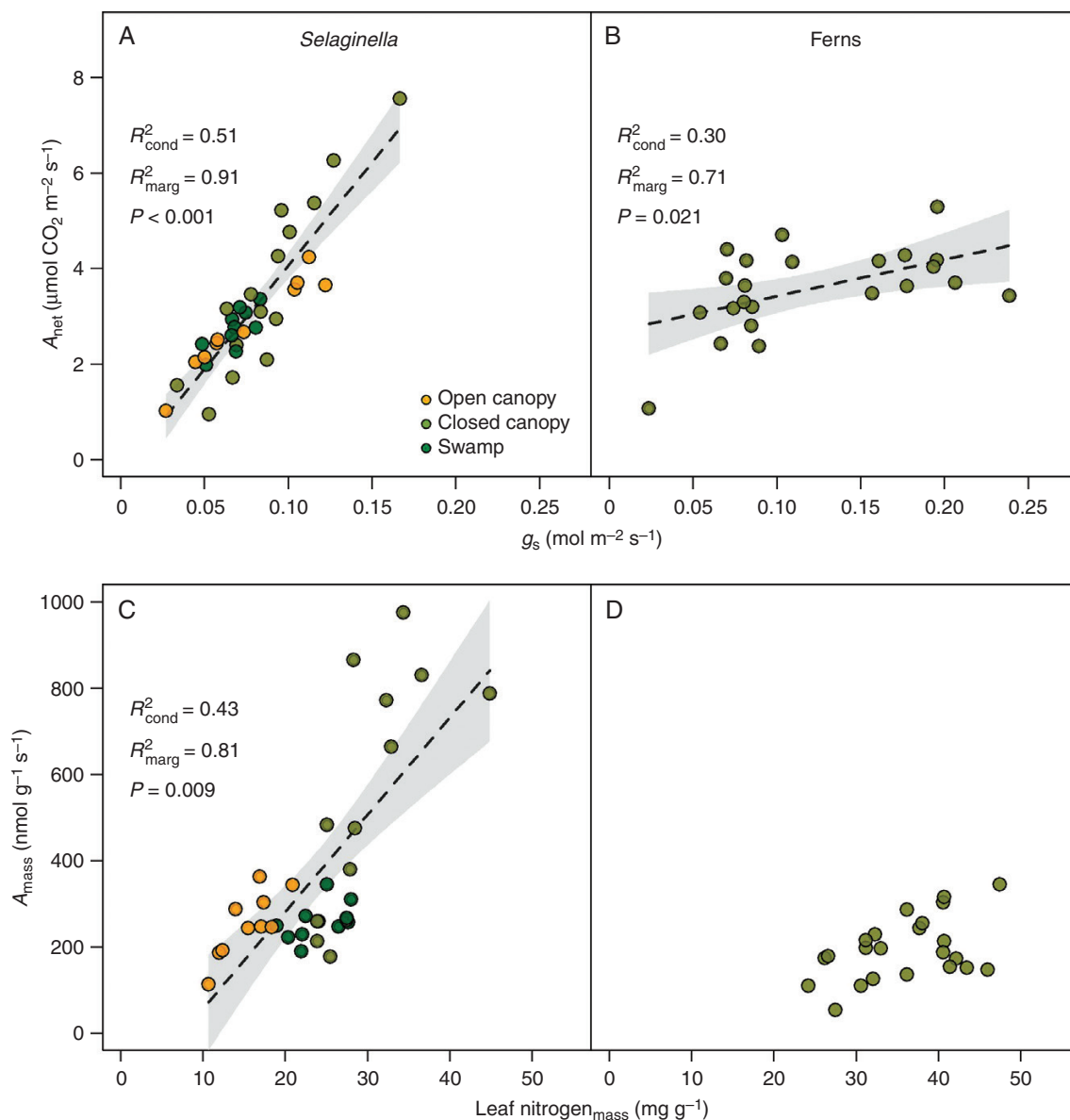


FIG. 4. Bivariate trait relationships across surveyed *Selaginella* species (A and C) and fern species (B and D) in a wet tropical forest. Colours represent habitats in which individuals occurred. For significant linear relationships, dashed lines represent model fits between traits and grey shaded areas are 95 % confidence intervals for the mean. Conditional and marginal R^2 (R^2_{cond} and R^2_{marg} , respectively) and P -values are reported for each significant linear model fit.

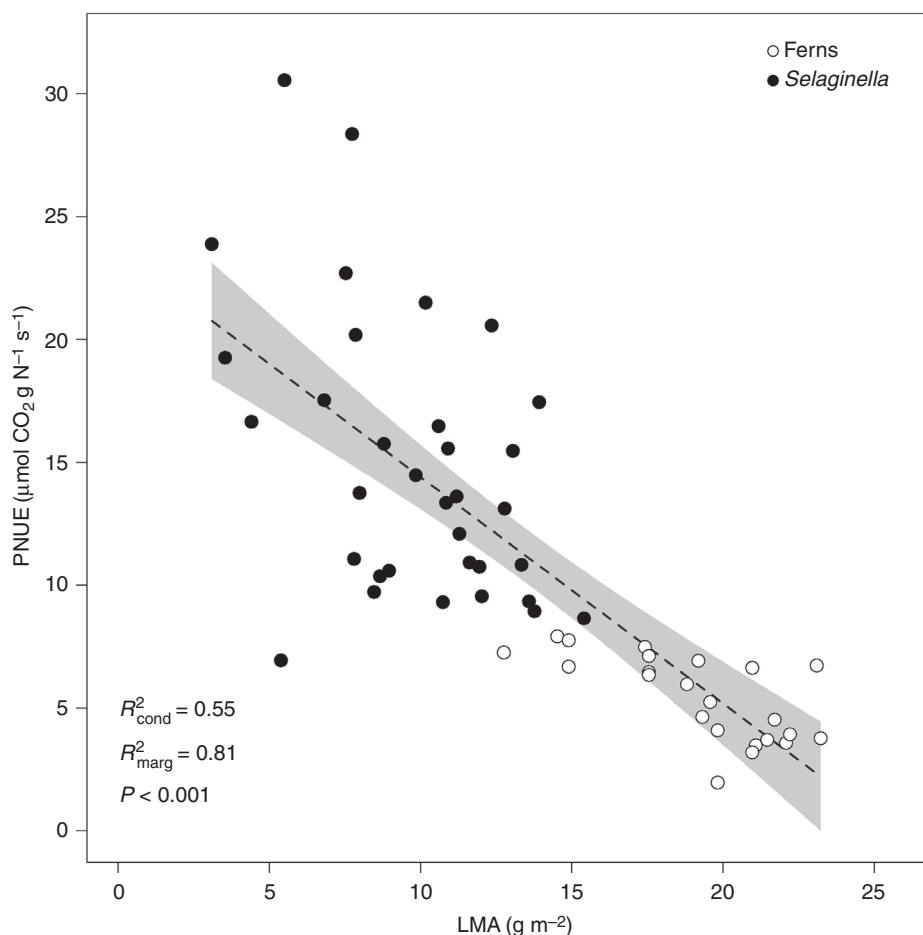


FIG. 5. Relationship between photosynthetic nitrogen use efficiency (PNUE) and leaf mass per area (LMA) across both *Selaginella* and fern taxa. The dashed lines represents the significant linear model fit for all measured individual plants (accounting for random effects of species) and grey shaded areas are 95 % confidence intervals for the mean. Conditional and marginal R^2 (R^2_{cond} and R^2_{marg} , respectively) and P -values are reported for the significant linear model fit.

(Brodribb *et al.*, 2007, 2009). Rates of g_s (approx. $0.08 \text{ mol m}^{-2} \text{ s}^{-1}$) were low, but also similar to reported rates ($0.06\text{--}0.19 \text{ mol m}^{-2} \text{ s}^{-1}$) of *Selaginella* species (Brodribb *et al.*, 2009; Ruzsala *et al.*, 2011). Predictably, photosynthesis was positively correlated with foliar N content and tightly coupled with g_s across all habitats. Overall, comparable leaf physiological function was detected across distinct forest floor habitats, despite species-level differences in A_n , g_s and WUE.

Trait selection can occur by habitat filter restrictions and microsite partitioning, leading to ecological sorting of species distributions (Reich *et al.*, 2003; Cornwell and Ackerly, 2009). Here, convergence of trait function occurred among *Selaginella* taxa within distinct habitats. Increased stomatal density was detected in taxa occupying open canopy habitats. Lycophytes probably exhibit passive stomatal control mechanisms (Brodribb and McAdam, 2011; Ruzsala *et al.*, 2011; McAdam and Brodribb, 2012a, b), and *Selaginella* taxa are shown to be particularly sensitive to increases in VPD (McAdam and Brodribb, 2013). Here, increases in the density of small-sized stomata may allow *Selaginella* taxa to better maintain plant water balance in open canopy habitats. Small and more dense stomata may have faster response times than larger stomata (Drake *et al.*, 2013), representing a possible mechanism for

Selaginella taxa to overcome inefficient stomatal responses to higher VPDs. Further work is needed, however, to confirm what specific environmental parameters drive shifts in stomatal traits among *Selaginella* taxa.

An established suite of plant traits allows species to succeed in light-limiting environments (Valladeres and Niinemets, 2008), which should be pronounced in tropical forest floors where low light can profoundly limit plant growth. Foliar N content of *Selaginella* taxa was higher in all closed canopy habitats compared with full sun habitats. Shifts in foliar N may be a simple consequence of microsite conditions, as low soil N has been detected in disturbed high-light tropical gaps (Bungard *et al.*, 2000). Alternatively, increased N investment in photosynthetic machinery in low-light habitats is a potential mechanism to improve light capture (Hikosaka and Terashima, 1995; Poorter and Evans, 1998). *Selaginella* taxa in swamp habitats were characterized by higher foliar chlorophyll *a* and *b* content and a lower LCP than in full sun habitats. Higher investment in chlorophyll *b* to increase absorption of blue light typically results in a lower chlorophyll *a*:*b* of understory plants (Boardman, 1977; Li *et al.*, 2018), a pattern that is partially supported in this study. Additionally, a low LCP in shade-adapted species allows for greater net carbon balance at low

light (Craine and Reich, 2005). Here, convergent trait strategies related to photosynthetic light capture appear to have increased the growth potential of *Selaginella* taxa established in heavily shaded tropical understorey habitats.

Functional ecological comparisons of *Selaginella* and ferns

The majority of surveyed species predominantly reside in low-light environments. Consequently, both plant groups should possess traits associated with resource retention and/or maximizing resource use efficiency (e.g. Reich *et al.*, 2003). Both genera exhibit predictable negative relationships with resource use efficiency and LMA, as seen with angiosperms (see Atkinson *et al.*, 2010; Renninger *et al.*, 2015; Reich and Flores-Moreno, 2017). The PNUE was similar to reported values for forest trees (approx. 1–12 $\mu\text{mol CO}_2 \text{ g N}^{-1} \text{ s}^{-1}$; Mediavilla *et al.*, 2001; Renninger *et al.*, 2015), despite having lower LMA in comparison. However, relationships between PNUE and retention (LMA) were functionally distinct between *Selaginella* and fern taxa (Fig. 5). Minimal overlap in PNUE between these two plant groups is likely to be a canalized evolutionary function of microphyll leaf morphology in lycophytes. Strategies for maximizing nutrient use appear centred around efficiency in *Selaginella* (low LMA of microphylls) compared with retention in ferns (higher LMA of megaphylls). Lycophyte leaf morphology is limited throughout their evolutionary history (Boyce, 2010), and data suggest that photosynthetic lycophyte microphylls can also have multi-year life spans (Callaghan, 1980). If true, high resource use efficiency combined with a long leaf life span suggests that diminutive *Selaginella* species may exist on a unique plane of the leaf economic spectrum (see Wright *et al.*, 2004).

Additionally, we show that fern and lycophyte taxa differ strongly in stomatal anatomical traits, despite being broadly equivalent in leaf carbon uptake. In overlapping habitats, stomata of ferns were larger and less dense than those of *Selaginella* species. Ferns growing in deep shade are typically characterized by large size stomata at low densities, with slow dynamic movement in humid conditions (Hetherington and Woodward, 2003). Overall, g_s was slightly higher in ferns (+13%), but g_s for both groups was relatively low. Under prevailing wet tropical forest conditions, prolonged water stress is unlikely, as WUE was similar between plant groups. Thus, strong selection pressure to maximize water use efficiency via functional shifts in stomatal anatomy appears unlikely. Stomatal size and density of *Selaginella* may be constrained by their smaller genome size (Beaulieu *et al.*, 2008), while stomata of ferns have also evolved to achieve wider apertures and reach higher rates of gas exchange than lycophytes under similar conditions (Franks and Farquhar, 2007). However, the observed infraspecific variation in stomatal density of *Selaginella* (see Fig. 3) may be a strategy to overcome these evolutionary constraints. Importantly, plasticity in stomatal density is not rare, and is commonly detected in plants exposed to elevated $[\text{CO}_2]$ treatments (Woodward, 1987; Gray *et al.*, 2000; Hetherington and Woodward, 2003; Franks and Beerling, 2009). Reich *et al.* (2003) note that trait differentiation among species occupying contrasting environments may be the result of trait divergence among plant lineages buried deep in evolutionary time. Here,

evolutionary driven trait divergence also drives adaptation of the ecophysiology of two plant lineages which overlap in tropical forest floor habitats.

Conclusion

Tropical forest understoreys are highly heterogeneous (Pearcy, 1983; Chazdon *et al.*, 1996), but trait-based analyses allow exploration of mechanisms that underpin species distributions in these complex environments. In this wet tropical forest floor, microclimate differences among habitats probably drove differential floristic composition of *Selaginella* taxa by filtering plant functional traits related to prevailing microclimate. Additionally, although it is common for *Selaginella* and fern taxa to overlap in tropical forest understoreys, these two groups exhibited contrasting strategies to optimize physiological function. These strategies appear to be related to evolutionary constraints associated with the independent evolution of micro- and megaphylls. Insight into leaf evolution and development focuses mainly within angiosperms, but key data are still needed in lycophytes and ferns to understand leaf evolutionary developmental of megaphylls (Vasco *et al.*, 2013). As ecological differences among vascular land plant species arise from different ways of acquiring the same resource (Westoby *et al.*, 2002), these results provide additional insights into how these early plant lineages make a living in an angiosperm world.

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