

Research paper

Extremely thin but very robust: Surprising cryptogam trait combinations at the end of the leaf economics spectrum



Tana Wuyun^a, Lu Zhang^{b,*}, Tiina Tosens^a, Bin Liu^a, Kristiina Mark^a, José Ángel Morales-Sánchez^a, Jesamine Jöneva Rikisahedew^a, Vivian Kuusk^a, Ülo Niinemets^{a,c,**}

^a Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 1, Tartu 51014, Estonia

^b College of Landscape and Architecture, Zhejiang A&F University, 666 Wusu Street, Hangzhou 311300, China

^c Estonian Academy of Sciences, Kohtu 6, Tallinn 10130, Estonia

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ABSTRACT

Leaf economics spectrum (LES) describes the fundamental trade-offs between leaf structural, chemical, and physiological investments. Generally, structurally robust thick leaves with high leaf dry mass per unit area (LMA) exhibit lower photosynthetic capacity per dry mass (A_{mass}). Paradoxically, “soft and thin-leaved” mosses and spikemosses have very low A_{mass} , but due to minute-size foliage elements, their LMA and its components, leaf thickness (LT) and density (LD), have not been systematically estimated. Here, we characterized LES and associated traits in cryptogams in unprecedented details, covering five evolutionarily different lineages. We found that mosses and spikemosses had the lowest LMA and LT values ever measured for terrestrial plants. Across a broad range of species from different lineages, A_{mass} and LD were negatively correlated. In contrast, A_{mass} was only related to LMA when LMA was greater than 14 g cm^{-2} . In fact, low A_{mass} reflected high LD and cell wall thickness in the studied cryptogams. We conclude that evolutionarily old plant lineages attained poorly differentiated, ultrathin mesophyll by increasing LD. Across plant lineages, LD, not LMA, is the trait that represents the trade-off between leaf robustness and physiology in the LES.

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1. Introduction

The leaf economics spectrum (LES) is a key concept in plant ecology linking leaf structure and function (Wright et al., 2004). It describes a set of interrelationships between leaf traits that are related to strategies in resource investment and return on investment, from acquisitive to conservative (Reich and Flores-Moreno, 2017). Several traits are considered key leaf economics traits, including leaf dry mass per unit area (LMA), leaf nitrogen (N_{mass}) and phosphorus (P_{mass}) contents per dry mass, maximum photosynthetic rate (A_{mass}), dark respiration rate per dry mass (R_{mass}),

and leaf life span. In general, plant species that employ an acquisitive strategy have higher fitness in habitats with more resources, and are mainly associated with higher A_{mass} , N_{mass} , P_{mass} and lower LMA. In contrast, species with a conservative strategy have the opposite suite of traits, which allow their persistence in resource-poor or stressful environments (Reich, 2014; Kunstler et al., 2016). Although it is well documented that LES relationships are valid across diverse habitats (Pan et al., 2020; Ishizawa et al., 2021), climates (Wright et al., 2004; Freschet et al., 2010), and life forms (Wright et al., 2004; Onoda et al., 2017), LES is mainly based on seed plants. Similarly, although some evolutionary trends have been investigated, these studies have only focused on seed plants (Shao et al., 2019; Liu et al., 2022). Non-seed plants, or cryptogams, such as bryophytes and lichens, contribute to 7% of net primary production and 50% of biological nitrogen fixation of terrestrial vegetation (Elbert et al., 2012), yet their spectrum of morpho-physiological traits remains understudied (Waite and Sack, 2010; Tosens et al., 2016; Allaby, 2019; Carriquí et al., 2019).

* Corresponding author.

** Corresponding author. Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 1, Tartu 51014, Estonia.

E-mail addresses: zhanglu@zafu.edu.cn (L. Zhang), ylo.niinemets@emu.ee (Ü. Niinemets).

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In classical LES theory, LMA traditionally serves as an indicator of leaf structural traits. Higher LMA usually indicates greater leaf robustness and is accompanied by lower N_{mass} and A_{mass} , which suggest a conservative resource usage strategy (Niinemets, 1999; Wright et al., 2004; Poorter et al., 2009; Reich and Flores-Moreno, 2017; Pan et al., 2020). The word “robustness” here means the quality of being strong and healthy, or less likely to break. However, accurate LMA values of cryptogams are difficult to measure. Cryptogams (especially bryophytes) tend to have ultrathin leaves that require detaching while counting; furthermore, high numbers of leaves are needed to ensure the accuracy of the dry weight measurement. In bryophytes, canopy mass per area (CMA) and shoot mass per projected shoot area (BMA) are more commonly used analogues of LMA (Waite and Sack, 2010; Wang et al., 2016; Carriquí et al., 2019; Grau-Andrés et al., 2022). Canopy mass per area is more of a bird-view area of a packed moss community, but the shoot area is a projected area of one single shoot. The one study that compared LMA and CMA of moss photosynthetic traits concluded that CMA is a more appropriate trait representing moss function (Waite and Sack, 2010; for other measurements of LMA in mosses, see Slate et al., 2017; Roos et al., 2019). Analogously, a recent study suggested that shoot level measurements in mosses provide limited insight (Grau-Andrés et al., 2022). Yet, mosses support the highest leaf area indices across plants worldwide (Niinemets and Tobias, 2019), implying that mosses have a great capacity for surface gaseous exchange, water absorption and trace gas and particulate matter deposition from atmosphere. These factors indicate that the shoot level functional traits of cryptogams (i.e., mosses) are worth investigating.

Trait-based ecology has expanded the traits examined in classical LES studies. For instance, studies that examine structural trade-offs underlying LES have included leaf thickness (LT), leaf density (LD), and mesophyll cell wall thickness (Onoda et al., 2011, 2017; Niinemets, 2015; Tosens et al., 2016). Because LD is calculated based on LMA and LT (Niinemets, 1999; Poorter et al., 2009), obtaining accurate LMA values in cryptogams is vital for derivation of accurate LD estimates, especially considering the complications of measuring LMA. To the best of our knowledge, no study has used a common leaf structural trait to integrate LES patterns of non-seed and seed plants.

Bryophytes, lycophytes, and ferns represent early land plants with their diversification dating back to more than 400 million years (Edwards et al., 1998; Banks, 2009). Bryophytes diverged already in early Ordovician, lycophytes in early Silurian, euphyllophyta in late Silurian (Donoghue et al., 2021) under harsh environments (Morris et al., 2018). Despite possessing less intricate structure and physiology (e.g., very limited capacity for physiological regulation of stomatal openness) compared to seed plants, they exhibit remarkable diversity and distribution and are often present in highly stressful habitats not suitable for seed plants (Banks, 2009; Turetsky et al., 2012; Tosens et al., 2016). Few studies have investigated LES traits of ferns, lycophytes, and/or bryophytes (Tosens et al., 2016; Wang et al., 2017; Carriquí et al., 2019). Studies have found that bryophytes, lycophytes, and ferns have low A_{mass} values (Tosens et al., 2016; Carriquí et al., 2019). This is paradoxical given that bryophytes do not appear to be robust. Determining the anatomical characteristics of bryophytes, lycophytes, and ferns, e.g., thickness of cell walls, is critical to gain mechanistic insight in this paradox.

In this study, we characterized the relationships between structural traits and other LES traits, particularly chemical and photosynthetic traits, across four lineages of cryptogams, i.e., liverworts, mosses, spikemosses, ferns. In addition, we examined the relationship between investment strategies and evolutionary history of land plants. Finally, we propose a standard protocol for measuring LMA in cryptogams.

2. Materials and methods

2.1. Plant material

We characterized various structural traits of 11 cryptogam species and two angiosperm species (Table S1 for detailed information of the plant material and growth conditions). The cryptogam species included *Plagiochila asplenioides* (L.) Dumort., *Marchantia polymorpha* L., *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr, *Plagiomnium undulatum* (Hedw.) T.J. Kop., *Selaginella moellendorffii* Hieron., *Selaginella uncinata* (Desv. ex Poir.) Spring, *Selaginella apoda* (L.) Spring, *Selaginella martensii* Spring, *Adiantum raddianum* C. Presl, *Dryopteris erythrosora* (D.C. Eaton) Kuntze, and *Nephrolepis cordifolia* (L.) C. Presl. The angiosperm species were *Cucumis sativus* L. and *Phaseolus vulgaris* L. For *Selaginella moellendorffii* and *Selaginella uncinata*, novel scientific names were reported as *Kungiselaginella moellendorffii* (Hieron.) Li Bing Zhang & X.M. Zhou and *Didiclis uncinata* (Desv. ex Poir.) Li Bing Zhang & X.M. Zhou, respectively (Zhou and Zhang, 2023). Although we used the names designated as “current names” at the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/taxonomy>), we will use the novel names in future work.

All plant materials were stabilized for at least two weeks under similar conditions with slight gradients in temperature (low–high), relative humidity (high–low), and light intensity (low–high) according to the relative divergence of each lineage (i.e., bryophytes, lycophytes, ferns, and seed plants). Growth conditions were meant to simulate favorable conditions for each lineage (Table S1). We attempted to maintain some similarities in growth conditions to allow for comparisons between distantly related plant lineages.

Bryophytes were collected from their natural habitats (Table S1). We acclimated three bryophytes (*P. asplenioides*, *S. ruralis*, *P. undulatum*) in the same growth chamber for seven months at 20/17 °C, relative air humidity of 80%, light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in a 12 h/12 h day/night cycle. *M. polymorpha* was obtained from a local greenhouse two weeks prior to experiments. We carefully removed plant material from the drain wall and placed it in a plastic box (20 cm × 10 cm × 10 cm) that we then covered before moving the plant material to the same plant growth chamber as the other bryophytes. The environmental conditions of the growth chamber were similar to those of a forest floor, where light intensity is rather low and the humidity rather high, with contrasting temperatures between day and night.

Two spikemosses (*S. moellendorffii* and *S. uncinata*) were propagated from plant parts, and were grown in 2-L plastic pots filled with a mixture of sand and commercial potting soil (1:1, v:v) (Biolan Oy, Finland) in a plant growth room at 23 °C, relative air humidity of 70%, light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in a 12 h/12 h day/night cycle for three months to allow new leaves to develop. The other two spikemosses (*S. apoda* and *S. martensii*) were obtained at the local market and put under the same conditions for two weeks before the experiment.

All fern species were obtained from the local market and acclimated at 25 °C, relative air humidity of 55–60%, light intensity of 100–130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in a 12 h/12 h day/night cycle. The two seed plants, *C. sativus* and *P. vulgaris*, were sowed with same pot and soil as for propagated spikemosses, and were grown at 25 °C, relative air humidity of 55–60%, light intensity of 140–200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in a 12 h/12 h day/night cycle. Plants were grown 3–4 weeks, or until they reached about 20–30 cm tall, with four to five fully expanded leaves, before being used in the experiments. All plants were watered every other day until soil field capacity was reached. All measurements were taken with at least three replicate plants.

2.2. Estimating leaf economics spectrum traits

According to different plant characteristics, two custom-made open gas-exchange systems with two-lines (reference and sample line) were used for the measurements. For bryophytes and lycophytes, a cylindrical 0.2 L chamber with an air flow rate of 1.48 L min^{-1} was designed to suit small structurally complex species (Morales-Sánchez et al., 2023). Light was evenly provided above the chamber by three dimmable LED lamps (Philips GmbH, Germany) with an intensity of $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The bryophyte and lycophyte were detached from their substrate and enclosed in the chamber. For ferns and seed plants, a double-walled 1.2 L cuvette was used at an air flow rate 1.75 L min^{-1} (Copolovici and Niinemets, 2010, for a detailed description). The chamber system was designed in a way that only the measured leaves/stems can be inserted in, to avoid possible contamination from soil gas. Four wide-beam halogen lamps (Osram GmbH, Germany) were used for light intensity of $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$. This light intensity was set according to previous work where the net assimilation rate of mosses increased within the range of $100\text{--}300 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (Slate et al., 2017). Previous work has also shown that the photo-inhibition limit for mosses is commonly above $480 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (Grau-Andrés et al., 2022). Thus, the design of a gradient of light acclimating condition lower than $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$, and a unified light intensity of $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$ while measurement was considered suitable for all plants in various lineages. Both measurements were taken under standard conditions of ambient CO_2 concentration of $390\text{--}400 \mu\text{mol mol}^{-1}$, humidity of $65\text{--}80\%$ and temperature of $23 \text{ }^\circ\text{C}$, controlled by circulating water through the double glass walls of the chambers. During plant enclosure, chamber CO_2 and H_2O concentrations were measured continuously using a LI-7000 $\text{CO}_2/\text{H}_2\text{O}$ analyzer (LI-COR Biosciences, USA).

Foliage (leaf, thallus or shoots) used in gas exchange measurements were flattened under a transparent glass and measured for their projected area. Foliage was then oven-dried at $70 \text{ }^\circ\text{C}$ for 48 h before the dry mass of plant material was recorded. Leaf nitrogen (N_{mass}), leaf phosphorus (P_{mass}), and leaf carbon (C_{mass}) contents per dry mass were determined with a Vario MAX CNS elemental analyzer (Elementar, Hanau, Germany). The N_{mass} , P_{mass} and C_{mass} contents of each sample were calculated as the percentage of corresponding element in the dry weight of the sample. Morphological differences between distantly related species introduced certain biases, due to various percentages of supporting material in leaf-like units of different plant lineages.

For surface area estimation in bryophytes and spikemosses, branches adjacent to those used for gas exchange measurements were harvested. The leaves (phyllids in mosses) were carefully removed, and their number was counted (about 500 leaves, more than 10 mg dry mass for each replicate measurements). Because the ultrathin leaves of bryophytes and spikemosses are light and easily dehydrated, the detached leaves were put on a piece of wet tissue. This tissue maintained the moisture of the leaves and prevented leaves from flying away due to disturbances caused by small movements. After fresh weight was registered, leaves were oven-dried at $70 \text{ }^\circ\text{C}$ for 48 h, and their dry masses were measured. Leaf dry to fresh mass ratio (D/F) was then calculated ($\text{D/F} = \text{leaf dry mass}/\text{leaf fresh mass}$). Six to seventeen (according to variation in leaf size) new leaves from each species were photographed with a stereo microscope (Olympus SZ40ESD, Tokyo, Japan) at $6.7\times$ to $10\times$ magnification. ImageJ 1.48v software (Wayne Rasband/NIH, Bethesda, MD, USA) was used for leaf area determination, and average leaf area was estimated as in Niinemets and Tobias (2019). The total leaf area per sample was found as the average leaf area multiplied by the number of leaves. Ultimately, leaf dry mass per

unit area (LMA) was estimated as the ratio of the dry mass of the sample to its corresponding total surface area. LMA was further used to estimate the dry mass of plant parts of bryophytes (except *M. polymorpha*) and spikemosses enclosed for the photosynthesis measurements and was used in A_{mass} calculation ($A_{\text{mass}} = A_{\text{area}}/\text{LMA}$). Leaf density (LD) was estimated as ($\text{LD} = \text{LMA}/\text{LT}$, see next section for estimation of leaf thickness, LT) (Niinemets, 1999; Poorter et al., 2009). Shoot mass (stem + leaves) per projected shoot area (BMA) was estimated to characterize the degree to which it differs from the true LMA (Niinemets and Tobias, 2019) (Table S2).

2.3. Microscopy and anatomical measurements

Leaf material was fixed in 2.5% glutaric aldehyde solution and subsequently infiltrated and embedded in resin according to a previous paper (Veromann-Jürgenson et al., 2020). Semi-thin cross-sections of $1 \mu\text{m}$ were prepared for light microscopy and ultra-thin sections of $70\text{--}90 \text{ nm}$ were prepared for transmission electron microscopy (TEM) using an ultramicrotome (Leica EM UC7, Leica Vienna, Austria). Precautions were taken to minimize tissue shrinkage, most importantly by employing a rapid dehydration protocol (dehydration was completed within 1.3 h) (Dykstra and Reuss, 2003). Leaf thickness (LT) was measured from sections stained with toluidine blue (Sigma Aldrich, USA) and viewed with brightfield EVOS FL Auto 2 Imaging System (ThermoFisher Scientific) at $40\times$ magnification. LT was measured from each section at 10 locations between the abaxial and adaxial cuticles according to a previous paper (Veromann-Jürgenson et al., 2017), then the average values of each plant replicates were used for further analysis. Sections for TEM were mounted on carbon-covered copper meshes (Electron Microscopy Sciences, Hatfield, PA, USA) and stained with uranyl acetate and lead citrate, before viewing with a Philips Tecnai 10 TEM (FEI, Eindhoven, The Netherlands) using an accelerating voltage of 80 kV and taken at $1900\times$ magnification. For measurements of cell wall thickness (CWT), cells were selected representing different tissue depth, and the average mesophyll cell wall thickness was calculated. CWT was measured at three fields of view and 10 locations along the cell wall from areas that had adjacent airspace, not attached to other cells (Tosens et al., 2016; Veromann-Jürgenson et al., 2017). Anatomical measurements were conducted in three replicate plants per species and from each plant 3–5 replicate sections were viewed.

2.4. Data analyses

All data shown in the figures and tables are presented as means \pm SE. In the correlation analyses, linear or nonlinear simulating functions were chosen to obtain the highest significant structure of the relationship. Range-dependent relationships were defined associated with the LMA value of 14 g m^{-2} , i.e., when clear correlations exist without a data point of $\text{LMA} < 14 \text{ g m}^{-2}$, while no correlation was found with these data points. In fact, 14 g m^{-2} is the lowest LMA value of terrestrial plants observed in the global database (Wright et al., 2004). In Table S2, all traits were compared between species using one-way ANOVA, followed by Duncan's test. Logarithmic data transformation was used when constructing heatmaps in Fig. 4. Analyses were conducted with OriginPro 2018. Statistical relationships were considered significant at $P < 0.05$. The thalloid liverwort *Marchantia polymorpha* differed from other bryophytes by having LT and LMA similar to angiosperms, but also having a larger non-photosynthetic tissue fraction and a low A_{mass} (Table S2), possibly because this species modified its structure to absorb more nutrition from salad factory drains where it grows. It was therefore considered as an outlier in some analyses.

3. Results

3.1. Leaf economics traits

The measured and estimated leaf economic traits are reported in Table S2. Although recent speciation is ongoing within old lineages, here we focus on their initial differentiation (Fig. S1). In bryophytes other than the thalloid liverwort *Marchantia polymorpha*, leaf dry mass per unit area (LMA) exhibited lowest values ever measured for terrestrial plants (6.8–11.9 g m⁻²), ca. 1-fold lower than the values observed in mesophytic annual angiosperms (17.2–21.5 g m⁻²); while shoot mass per projected shoot area (BMA) showed extremely high values (70.8–88.7 g m⁻²), ca. 3-fold

higher than values observed in angiosperms (Table S2). Leaf thickness (LT) was ca. 6-fold lower in bryophytes (18.5–23.3 μm) than in angiosperms (120–180 μm; Table S2), whereas leaf density (LD) was ca. 2-fold greater in bryophytes (0.35–0.65 g cm⁻³) than in angiosperms (0.12–0.15 g cm⁻³; Table S2). Cell wall thickness (CWT) was about an order of magnitude greater in bryophytes (1–2 μm) than in seed plants (0.14–0.18 μm; Table S2). Photosynthetic capacity of seed plants was the highest on leaf mass (A_{mass}), area (A_{area}), leaf N basis compared to that of other lineages (PNUe, Table S2). Nitrogen (N_{mass} and N_{area}) and phosphorus (P_{mass} and P_{area}) contents increased from early-diverged plant lineages to more recent plant lineages. Mass-based carbon content (C_{mass}), in contrast, was lower in seed plants (Table S2).

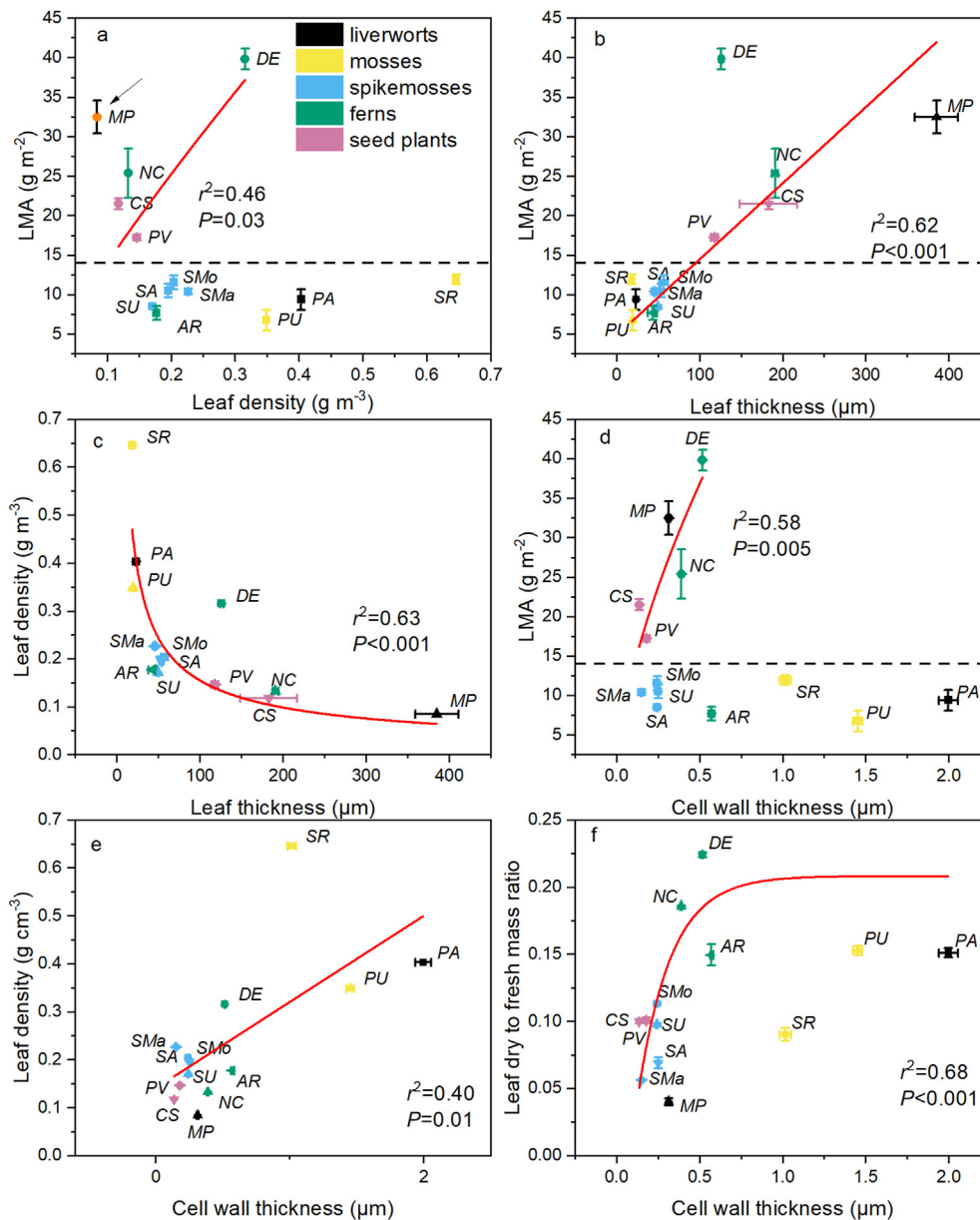


Fig. 1. Bivariate relationships between leaf structural traits. (a) LMA vs leaf density. (b) LMA vs leaf thickness. (c) Leaf density vs leaf thickness. (d) LMA vs cell wall thickness. (e) Leaf density vs cell wall thickness. (f) Leaf dry to fresh mass ratio vs cell wall thickness. In (a), (b), and (d), the dashed line indicates 14 g m⁻² in LMA. PA, *Plagiochila asplenioides*; MP, *Marchantia polymorpha*; SR, *Syntrichia ruralis*; PU, *Plagiommium undulatum*; SMO, *Selaginella moellendorffii*; SU, *Selaginella uncinata*; SA, *Selaginella apoda*; SMa, *Selaginella martensii*; AR, *Adiantum raddianum*; DE, *Dryopteris erythrosora*; NC, *Nephrolepis cordifolia*; CS, *Cucumis sativus*; PV, *Phaseolus vulgaris*. The outlier in (a) is colored orange and indicated with arrows. Error bars indicate ± SE (n = 3). Error bars are not presented for leaf density (LD) in all related panels, because LD was calculated based on average of LMA and leaf thickness from different leaves. Non-linear relationships in form of $y = ax^b$ were used to fit the data in (a), (c), and (d), and $y = a \exp(x/t) + y_0$ was used to fit the data in (f). Linear regressions were used to fit the data in (b) and (e). In (b), no relationship was found in the range of LMA > 14 g m⁻². Statistical relationships were considered significant at P < 0.05.

3.2. Relationships among leaf structural traits

In bivariate structural relationships, LMA-range-dependent patterns were observed in relationships with LD and CWT; when LMA values were in the higher range ($> 14 \text{ g m}^{-2}$), LMA was weakly but positively related to LD (Fig. 1a) and CWT (Fig. 1d). In the lower LMA range ($< 14 \text{ g m}^{-2}$), and for all data pooled, LMA was weakly related to LES structural traits (Fig. 1a–d). A positive correlation was found between LMA and its component LT (Fig. 1b), whereas LT and LD were exponentially negatively correlated (Fig. 1c). LD and leaf dry to fresh mass ratio (D/F) were positively related to CWT (Fig. 1e and f).

3.3. Relationships between leaf chemical with structural traits

LMA did not show a clear relationship to N_{mass} (Fig. 2a) or P_{mass} (Fig. 2b). LT was positively related to N_{mass} (Fig. 2c) and P_{mass} (Fig. 2d). Negative relationships of LD (Fig. 2e and f), CWT (Fig. 2g, h), and D/F (Fig. S2) with N_{mass} and P_{mass} were observed with similar clustering of taxonomic groups as with LT. Bryophytes and ferns were clustered near the lower end, whereas spikemosses and seed plants were clustered near the higher end of the nitrogen and phosphorus gradients (Fig. 2).

3.4. Relationships between leaf photosynthetic and structural traits

A strong positive relationship was found between A_{mass} and N_{mass} . Again bryophytes and ferns were clustered near the lower end of the relationship, whereas spikemosses and seed plants were clustered near the higher end (Fig. 3a). Range-dependent patterns were again observed in relationships with LMA and A_{mass} ; when LMA values were $> 14 \text{ g m}^{-2}$, it was negatively related to A_{mass} (Fig. 3b). Except for the positive correlation of A_{area} with LT (Fig. 3c), A_{mass} relationships with other structural traits were negative (Fig. 3d–f). In the relationships between A_{mass} and D/F, ferns and seed plants, bryophytes (including liverworts and mosses) and spikemosses were grouped separately to form two different relationships (Fig. 3f).

4. Discussion

Our study presents leaf economics spectrums (LES) for 13 species belonging to five evolutionarily different plant lineages. Our findings indicate classical LES theory should be modified. Specifically, leaf dry mass per unit area (LMA) should not be considered a universal trait for leaf structural robustness. Instead, leaf density (LD) should be used, especially when comparing newer lineages to evolutionarily older lineages. In addition, we propose that plant lineages that diverged earlier employ conservative resource usage strategies, whereas plant lineages that diverged more recently employ acquisitive resource usage strategies.

4.1. LMA blurs bivariate relationships among leaf economics traits

In classical LES theory, leaf robustness is represented by high LMA values, accompanied by lower N_{mass} and A_{mass} , suggesting a conservative resource usage strategy (Wright et al., 2004; Reich and Flores-Moreno, 2017; Pan et al., 2020). Here, we did not find clear relationships between LMA and LES traits such as N_{mass} (Fig. 2a) or A_{mass} (Fig. 3b), indicating that the classical morpho-physiological trade-offs between LMA and $N_{\text{mass}}/A_{\text{mass}}$ do not apply to all plant lineages. This discrepancy with previous work is probably because previous studies relied on databases composed of species with high LMA values ($> 14 \text{ g m}^{-2}$) (Wright et al., 2004; Tosens et al., 2016; Veromann-Jürgenson et al., 2017; Onoda et al., 2017; Carriquí et al.,

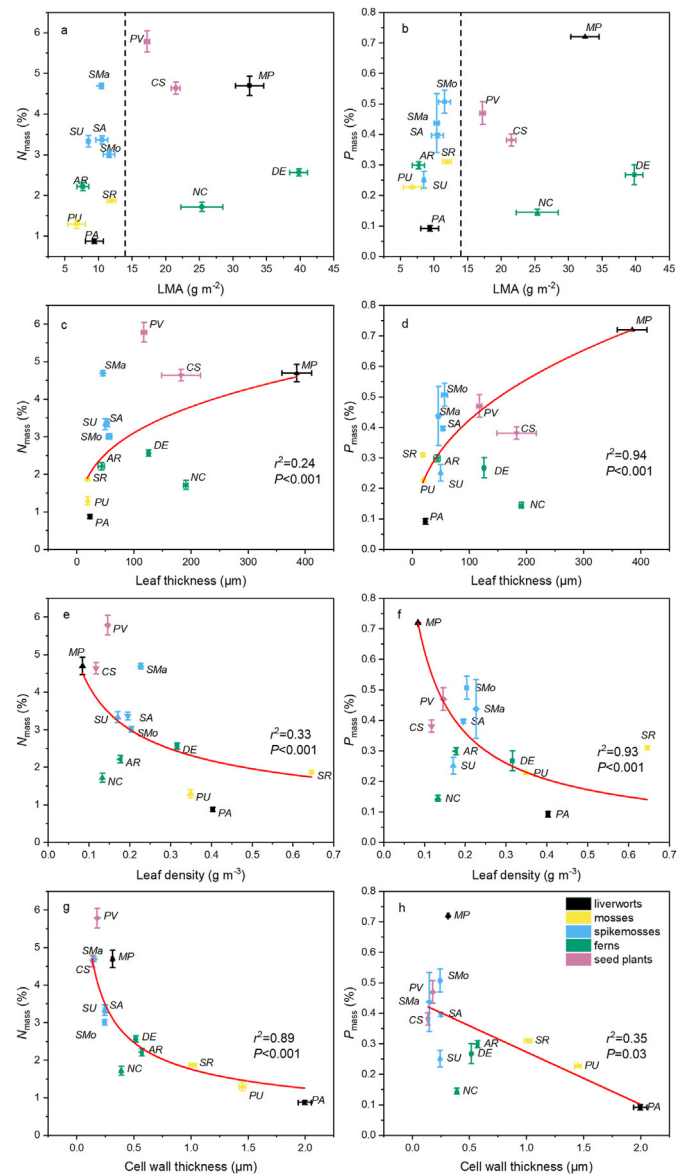


Fig. 2. Bivariate relationships between leaf chemical and structural traits. (a) N_{mass} vs LMA. (b) P_{mass} vs LMA. (c) N_{mass} vs leaf thickness. (d) P_{mass} vs leaf thickness. (e) N_{mass} vs leaf density. (f) P_{mass} vs leaf density. (g) N_{mass} vs cell wall thickness. (h) P_{mass} vs cell wall thickness. Error bars indicate \pm SE ($n = 3$). Error bars are not presented for leaf density (LD) in all related panels, because LD was calculated based on average of LMA and leaf thickness from different leaves. Species abbreviations as in Fig. 1. Non-linear relationships in form of $y = ax^b$ were used to fit the data in (c–g). Linear regression was used to fit the data in (h).

2019). Cryptogams, which have lower LMA values, have seldomly been included in these types of studies (Wright et al., 2004; Wang et al., 2017; Carriquí et al., 2019). Although a few studies have reported small LMA values in bryophytes (Waite and Sack, 2010; Slate et al., 2017; Roos et al., 2019), the biological function of small LMAs in this functional type has been neglected; furthermore LMAs of bryophytes have yet to be correlated with other traits. In the current study, bivariate LMA relationships with LD and CWT showed range-dependent patterns (Fig. 1a and d). When LMA values are in the higher range ($> 14 \text{ g m}^{-2}$), LES relationships are consistent with classical LES theory (Wright et al., 2004). LMA relationships with A_{mass} (Fig. 3b) were also range-dependent. The negative relationships, within the higher range of LMA values, are consistent with findings that higher LMA is associated with longer lived

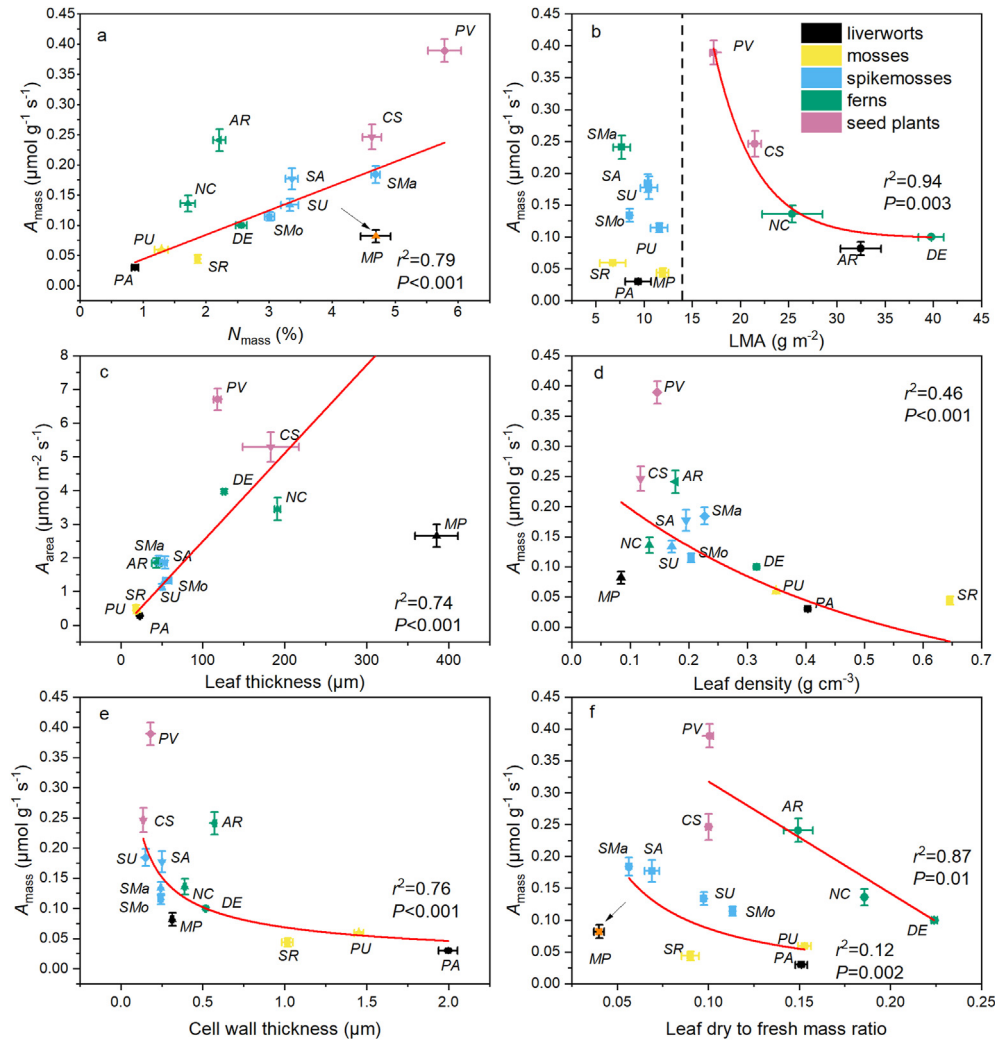


Fig. 3. Bivariate relationships between A_{mass} and other functional traits. (a) A_{mass} vs N_{mass} . (b) A_{mass} vs LMA. (c) A_{area} vs leaf thickness. (d) A_{mass} vs leaf density. (e) A_{mass} vs cell wall thickness. (f) A_{mass} vs leaf dry to fresh mass ratio. The outliers in (a) and (f) are colored orange and indicated with arrows. Error bars indicate \pm SE ($n = 3$). Error bars are not presented for leaf density (LD) in the related panel, because LD was calculated based on average of LMA and leaf thickness from different leaves. Species abbreviations as in Fig. 1. Non-linear relationships in form of $y = a \exp(-x/t) + y_0$ was used to fit the data in (b) and (d), and $y = ax^b$ were used to fit the data in (e) and (f, lower line). In (f), ferns and seed plants, bryophytes and spikemosses were fitted separately. Linear regressions were used to fit the data in (a) and (f upper line).

photosynthetic organs and lower A_{mass} in both herbaceous and woody species (Poorter et al., 2009).

In plants with low LMA values ($< 14 \text{ g m}^{-2}$), the relationship between LMA and A_{mass} was not statistically significant, similar to previous studies of 10 Hawaiian moss species (Waite and Sack, 2010) and 15 boreal mosses (Grau-Andrés et al., 2022) that showed these traits were not directly related. Indeed, when data from the current study were integrated with global data for terrestrial plants, the correlation between LMA and A_{mass} was stronger when plants with smaller LMA ($< 14 \text{ g m}^{-2}$) were excluded (Fig. S3). However, for species with LMAs smaller than 14 g m^{-2} , larger sample sizes may be needed to test if they still show similar negative relationships between LMA and traits that indicate more acquisitive features (e.g., N_{mass} and A_{mass}). Aquatic plants, such as duckweed (LMA $< 14 \text{ g m}^{-2}$), are believed to utilize a highly acquisitive resource usage strategy (Ishizawa et al., 2021). Interestingly, our analysis indicates that the LES relationships of cryptogams are distinct from those of duckweed (Fig. S3), indicating that the small LMA values of duckweed are fundamentally different from those of land plant species belonging to

evolutionarily older lineages. One explanation for this distinction is that leaf density is lower in duckweeds than in the bryophytes and lycophytes of this study because duckweeds have thicker leaves (a magnitude of $100 \mu\text{m}$) (Jansen et al., 2001).

We speculate that investigating the true LMA of species from evolutionarily older groups will help augment classical LES theory. Using deviated data points of LMA values in evolutionarily older lineages (i.e., plant lineages with low LMA values) will lead to extremely high estimated values of LD. For example, previous studies reported that the LMA of *Selaginella martensii* is 54.7 ± 5.4 (Carricú et al., 2019); in contrast, our study found it to be 10.39 ± 0.34 (Table S2). Overestimations of this magnitude lead to wildly inaccurate LD estimates, e.g., the leaves of *Selaginella martensii* would be estimated as denser than iron. Clearly, a consistent methodology is needed to measure LMA, particularly for species from evolutionarily older lineages. Nevertheless, the unclear relationships between LMA with other functional traits suggest that when non-seed plants are involved, LMA alone is not a reliable trait for determining plant investment strategy.

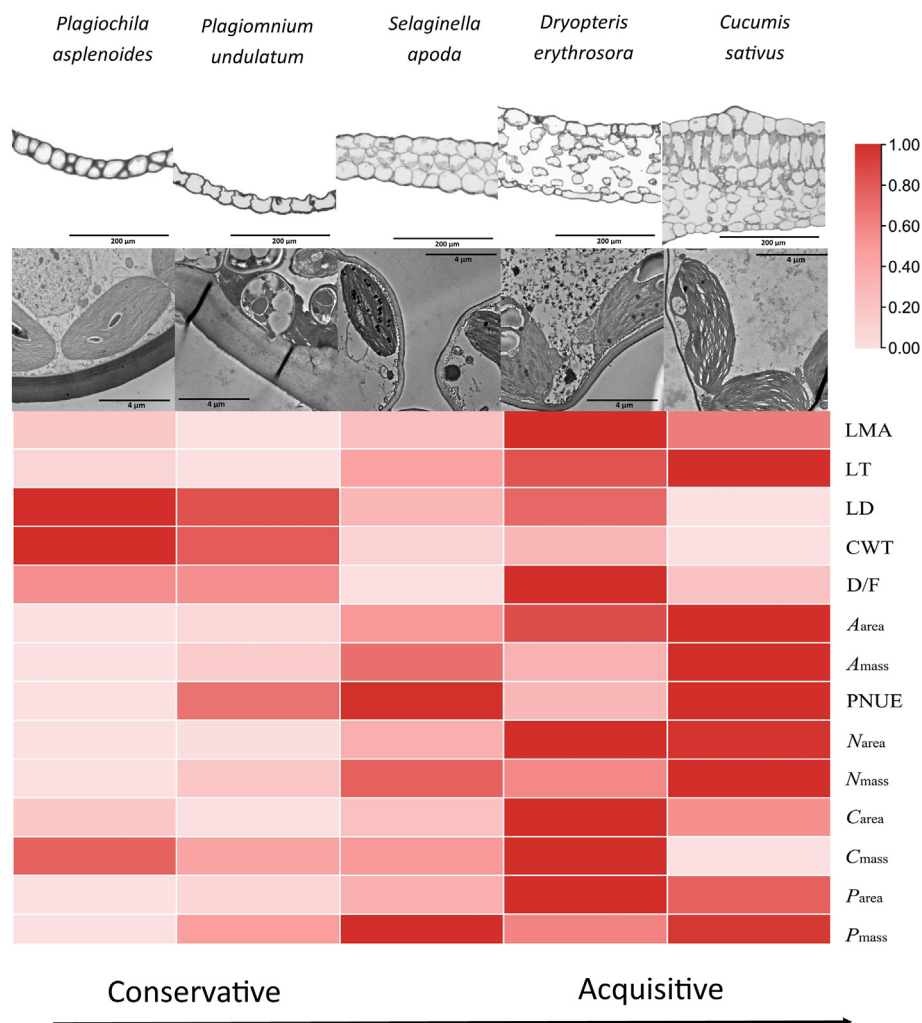


Fig. 4. Images of semithin (a) and ultrathin transmission electron microscopy cross-sections (b), and heatmap of leaf economic traits values (c) in five representative species. Heatmap represents the $\log(x+1)$ -transformed values of all traits. LMA, leaf dry mass per unit area; LT, leaf thickness; LD, leaf density; CWT, cell wall thickness; D/F, leaf dry to fresh mass ratio; A_{area} , area-based photosynthesis; A_{mass} , mass-based photosynthesis; PNUE, photosynthetic nitrogen use efficiency; N_{area} , nitrogen per area; N_{mass} , nitrogen per mass; C_{area} , carbon per area; C_{mass} , carbon per mass; P_{area} , phosphorus per area; P_{mass} , phosphorus per mass. The hotter color represents higher values and cooler ones represents lower values. Row scale was performed (scale method ZeroToOne) to visualize patterns of each trait along with evolution time.

4.2. Leaf density is a better trait to reveal interrelationships among leaf economics traits

Studies on LD and LT (two components of LMA) are limited. High LT values typically imply thicker layers of photosynthetic mesophyll cells per unit area, and accordingly greater photosynthetic capacity per unit area (Niinemets, 2001; Poorter et al., 2009). In the present study, LT showed a clear range-independent, positive correlation with LMA (Fig. 1b) across species, and it was also positively correlated with N_{mass} (Fig. 2c), P_{mass} (Fig. 2d), and A_{area} (Fig. 3c). These results are consistent with other studies (Xiong and Flexas, 2018), indicating that LT is an unambiguous trait that can be used to compare resource usage strategies of species across an evolutionary timescale, i.e., evolutionarily older vs. newer lineages. Because higher LT usually corresponds to more layers of photosynthetically active cells and larger storage capacity for photosynthetic capabilities (Niinemets, 2001; Poorter et al., 2009), it is not surprising that LD was negatively correlated with LT when all lineages were pooled in the current study (Fig. 1c, $r^2 = 0.63$, $P < 0.001$). Although LT is largely neglected in classical LES theory, high LT values are often associated with higher LMA, which is an indicator of leaf structural

robustness (Wright and Cannon, 2001; Blonder et al., 2011). However, in the few studies that have examined the relationship between LD and LT, variance in LMA across species (mainly seed plants) can be more readily explained by LD (80%) than by LT (20%) (Poorter et al., 2009).

Evidence indicates that cell walls account for a significant percentage of leaf dry mass, e.g., 20–60% (Read and Sanson, 2003) or 37–69% (Kitajima et al., 2012). In our study, LD was positively correlated with CWT, indicating that greater cell wall investment leads to larger density (Fig. 1e). Analogously, LD and CWT are both negatively correlated with chemical traits (e.g., N_{mass} and P_{mass}) (Fig. 2e–h) and physiological traits (e.g., A_{mass}) (Fig. 3d and e). This finding indicates that species with higher LD and CWT tend to invest less in the photosynthetic apparatus, further suggesting that plant investment in supportive structure can directly influence how much they can invest in photosynthetic structure, i.e., a trade-off between structural and metabolic traits (Wright et al., 2004; Shipley et al., 2006).

Nitrogen and phosphorus are essential for plant physiological processes such as photosynthesis. Previous studies have mainly focused on the relationship between nitrogen content and net

assimilation rate (Carriquí et al., 2019), as well as on the highly studied traits such as LMA (Tosens et al., 2016; Onoda et al., 2017). Few studies have explored the relationships between structural traits, such as LT, LD, CWT and D/F, and leaf chemical traits. There is a well-established trade-off between the nitrogen investment to leaf cell wall mass and photosynthetic apparatus (Onoda et al., 2017). In the present study, we found that N_{mass} and P_{mass} showed clear and similar relationships with each structural trait (Fig. 2c–h). In general, plant leaves capable of absorbing and conserving more chemical nutrition are less structurally robust (Reich, 2014). This fits well with our current conclusion that higher levels of nutrient content indicate a better acquisitive strategy in nature. Relationships between photosynthesis and structural traits have been previously examined (Carriquí et al., 2019; Flexas et al., 2021), however, CWT was only found to be negatively correlated with photosynthetic rate A_{mass} and A_{area} . In our study, A_{mass} was positively correlated with LT (Fig. 3c), but negatively correlated with LD, CWT and D/F (Fig. 3d–f).

4.3. Evolution of leaf economics trait combinations

While we acknowledge that speciation has been ongoing in every plant lineage, e.g., spikemoss species (Zhou and Zhang, 2023), in this study we examine evolutionary history of plant lineages in terms of their initial divergence. Non-seed plants are known to have diverged earlier than seed plants (Morris et al., 2018). Furthermore, previous research suggests that phylogenetic relatedness accounts for ca. 50% of the total variance at the family level but not at the species level (Shao et al., 2019). In our study, non-seed plants, with fewer layers of cells than seed plants, were shown to be structurally robust (Fig. 4 and S4), with high CWT, LD and C_{mass} , yet with low LT and LMA (Fig. 4). Previous studies have shown that photosynthetic organs in non-seed plants are thin (Tosens et al., 2016; Veromann-Jürgenson et al., 2017; Carriquí et al., 2019); thus, it is plausible that evolutionarily older groups (bryophytes, lycophytes, and some ferns, Fig. S1) invest resources in CWT and LD to build more robust cell structures, while sacrificing leaf storage capacity by forming extremely thin (i.e., low LMA) leaves. Evolutionarily newer plants, e.g., seed plants (Morris et al., 2018; Fig. S1), invest resources in building photosynthetically active mesophyll layers instead of protective structures. For instance, most of the mesophyll cell volume of seed plants is occupied by vacuoles, which are less dense than organelles like chloroplasts or lignified tissues like the cell wall (Poorter et al., 2009). Consequently, LD is generally smaller in seed plants than in non-seed plants (e.g., bryophytes have one-cellular layer leaves, which have virtually no vacuoles and extremely thick cell walls). In non-seed plants (e.g., resurrection plants), high CWT allows plants to tolerate low water potentials without cell collapse (Pandey et al., 2010; Roberts et al., 2012; Niinemets et al., 2018). These plants regularly sustain very low water potential due to changes in tissue water content, which is different for plants that control water loss through stomata.

The cluster of plant lineages in various bivariate relationships is informative. For example, we found that A_{mass} is positively correlated with N_{mass} , consistent with previous findings (Wright et al., 2004). Specifically, N_{mass} was lowest in bryophytes, followed by ferns and lycophytes, then seed plants (Fig. 2a). This finding indicates that evolutionarily older plant groups contain less nitrogen, which supports better acquisitive features. These results seem to indicate that the ability to absorb nitrogen depends on the development of root or other vascular organs. Evolutionarily older plants have either no vascular tissue or lack fully functional vascular tissue (Wang et al., 2017). Furthermore, clustering patterns in bivariate relationships between most of the functional traits (Figs. 1–3)

suggest that plants evolved from more recent lineages, i.e., seed plants, tend to have thicker leaves, less density, thinner cell walls and higher water content than older ones. These trait combinations indicate that evolution has directed plant divergence from employing conservative strategies to acquisitive ones in general. This is in line with previous reports that showed bryophytes have resource-conservative trait spectra characterized by water-holding capacity and photosynthetic traits at the canopy level (Waite and Sack, 2010; Grau-Andrés et al., 2022).

5. Conclusion

Leaf economics modelling currently lacks data on evolutionarily older lineages such as bryophytes. These simple-structured plants provide great insight into the different roles LES traits play in strategies employed by plants, thus, expanding our understanding of how plants invest resources. In this study we characterized LES and associated traits in unprecedented details in cryptogams covering five evolutionarily different lineages. We also developed a new protocol for measuring LMA in cryptogams. Our results link plant traits responsible for structural robustness and traits responsible for overall physiological activity in a broad range of species from different lineages. We found that relationships between leaf traits and LMA were range-dependent. Specifically, higher LMA values shared relationships with other leaf traits in ways consistent with classical LES; in plants with smaller LMA values, e.g., bryophytes and lycophytes, these relationships were not found. In addition, we propose that LD may serve as a common indicator of leaf structural features covering at least five evolutionarily different plant lineages. In the future, broader taxa should be included to obtain a more precise relationships between functional traits.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

CRediT authorship contribution statement

Tana Wuyun: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Lu Zhang:** Writing – original draft, Supervision, Funding acquisition, Data curation. **Tiina Tosens:** Resources, Methodology, Investigation. **Bin Liu:** Resources, Methodology, Data curation. **Kristiina Mark:** Resources, Methodology, Data curation. **Jose Angel Morales Sanchez:** Resources, Methodology. **Jesamine Jöneva Rikisahedew:** Methodology. **Vivian Kuusk:** Writing – review & editing, Methodology. **Ülo Niinemets:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pld.2024.04.009>.

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