

Comparative anatomy of leaf petioles in temperate trees and shrubs: the role of plant size, environment and phylogeny

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- **Background and Aims** Petioles are important plant organs connecting stems with leaf blades and affecting light-harvesting ability of the leaf as well as transport of water, nutrients and biochemical signals. Despite the high diversity in petiole size, shape and anatomy, little information is available regarding their structural adaptations across evolutionary lineages and environmental conditions. To fill this knowledge gap, we investigated the variation of petiole morphology and anatomy of mainly European woody species to better understand the drivers of internal and external constraints in an evolutionary context.
- **Methods** We studied how petiole anatomical features differed according to whole-plant size, leaf traits, thermal and hydrological conditions, and taxonomic origin in 95 shrubs and trees using phylogenetic distance-based generalized least squares models.
- **Key Results** Two major axes of variation were related to leaf area and plant size. Larger and softer leaves are found in taller trees of more productive habitats. Their petioles are longer, with a circular outline and are anatomically characterized by the predominance of sclerenchyma, larger vessels, interfascicular areas with fibres and indistinct phloem rays. In contrast, smaller and tougher leaves are found in shorter trees and shrubs of colder or drier habitats. Their petioles have a terete outline, phloem composed of small cells and radially arranged vessels, fibreless xylem and lamellar collenchyma. Individual anatomical traits were linked to different internal and external drivers. Petiole length and vessel diameter increase with increasing leaf blade area. Collenchyma becomes absent with increasing temperature, and petiole outline becomes polygonal with increasing precipitation.
- **Conclusions** We conclude that species' temperature and precipitation optima, plant height, and leaf area and thickness exerted a significant control on petiole anatomical and morphological structures not confounded by phylogenetic inertia. Species with different evolutionary histories but similar thermal and hydrological requirements have converged to similar petiole anatomical structures.

Key words: Leaf area, petiole length, petiole anatomy, vessel diameter, supportive cells, temperate trees, temperate shrubs.

INTRODUCTION

Petioles are one of the most efficient structures in plants and represent an essential connection between the stem and the plant's photosynthetic machinery, the leaf blade (Niinemets and Fleck, 2002; Faisal *et al.*, 2010; Levionnois *et al.*, 2020). The primary function of a petiole is to provide mechanical support to self-hold and adjust leaf position towards the sun, improving light-harvesting ability. Apart from leaf position, they also play a key role in the hydraulic pathway throughout the plant, transporting water, nutrients and biochemical signals to the leaves, and photosynthates and other products towards the shoot (Niinemets and Fleck, 2002). Commonly green, and thus photosynthetically active, petioles may be stiff or flexible, long or

short, but are usually wider at their base. In some woody taxa, they also can activate an abscission zone that ensures proper leaf shedding at senescence. Similar to variation of leaf blade size (Wright *et al.*, 2017), the wide variation of petiole size, toughness and lifetime among plants must be considered when specific metabolic activities are evaluated. Vapour loss and hydraulic conductivity, for instance, change not only among species but also within the same taxa and are dependent on leaf position, sun exposure and vascular features (Sack *et al.*, 2005; Poorter and Rozendaal, 2008).

Although petioles and leaves are highly diverse in size, shape and hence anatomical settings, comparative studies linking petiole structures with leaf parameters or whole-plant size across species and evolutionary lineages are rare. Most studies

involve only one or a few closely related species (Niklas, 1992; Aasamaa and Söber, 2010; Brocious and Hacke, 2016; Mahley et al., 2018), which provide a narrow view of potential variation. Petiole anatomical studies are mainly descriptive, focusing on the characterization of distinct taxa (Metcalf and Chalk, 1950) or solving specific taxonomic problems (Ganem et al., 2019; Palacios-Rios et al., 2019; Karaismailoğlu, 2020). Nevertheless, anatomical traits are frequently combined with ecophysiological aspects (Niklas, 1992; Tadriss et al., 2014). Information about petiole biomechanics is commonly used to explain relationships between structure and anatomy, such as the size of epidermal cells and cross-sectional geometry compared with the size of the leaf blade, petiole stiffness, bending capacity or leaf angle (Niinemets and Fleck, 2002; Faisal et al., 2010; Levionnois et al., 2020). Additionally, distinct xylem traits such as vessel number, diameter and length have been used to examine physiological mechanisms of leaf hydraulic potential and vulnerability to xylem cavitation in petioles (Hacke and Sauter, 1996; Coomes et al., 2008; Aasamaa and Söber, 2010; Hochberg et al., 2014; Brocious and Hacke, 2016; Gebauer et al., 2019).

Little information is available about the relative importance of internal (leaf and plant size) and external climatic (temperature, precipitation) drivers for interspecific variation in petiole anatomical and morphological structures. To our knowledge, only six studies providing an ecological interpretation that combines petiole morphoanatomy of one or a few species with abiotic conditions such as desiccation, light availability and wind gradient are available (Niklas, 1992; Hacke and Sauter, 1996; Niinemets and Fleck, 2002; Abrantes et al., 2013; Klepsch et al., 2016; Louf et al., 2018). Likewise, it is well known that large-statured and large-leaved plants predominate in wet, warm and productive environments, while short-statured and small-leaved plants occur in cold or arid environments, such as at high latitudes and elevations (Wright et al., 2017), but little is known about variation in petiole anatomy across these environmental and plant size gradients (Baird et al., 2021). Concerning vessel diameter variation, for instance, two explanations seem to complement each other, based on studies of roots, stems and branches (e.g. Zimmermann and Potter, 1982; Alder et al., 1996; Hacke and Sauter, 1996; Martinez-Vilalta et al., 2002). One hypothesizes that the variation in vessel diameter reflects environmental constraints operating at the whole-plant level (Hacke et al., 2017), while the other relates increasing vessel diameter along the plant stem to provide sap conduction requirements (Olson et al., 2013). However, to our knowledge, there are no studies that have combined petiole traits with whole-plant size, leaf traits and environmental factors in a phylogenetic context.

To fill this knowledge gap on petiole morphoanatomical trait variation and to better understand internal and external drivers and constraints in an evolutionary context, by using phylogenetic comparative models we investigated the variation of petiole morphology and anatomy among a census of mainly European woody species growing in contrasting environmental conditions. In particular, we studied how petiole anatomical features differed according to whole-plant size, leaf traits, thermal and hydrological conditions, and taxonomic origin for a wider group of temperate trees and shrubs. The study is based on 95 woody species and 18 morphoanatomical features, such as cross-sectional geometry, epidermis traits, and conductive, mechanical and storage tissue structures. Given that the leaves

are the main photosynthetic apparatus of plants and that the petioles have a major role to support them mechanically and functionally, we assumed that the structure of the petiole will be more affected by leaf characteristics than by abiotic factors. We also expected that the supporting tissues (collenchyma and sclerenchyma) would be more developed in the larger leaves to optimize the ability to sustain leaves. Concomitantly, due to the effect of leaf size variation and hydraulic architecture being directly reflected in photosynthetic and water transport efficiency (Givnish, 1988; Terashima et al., 2011; Sack et al., 2012; Scoffoni et al., 2016), we expected vessel diameter to increase with leaf blade area.

MATERIALS AND METHODS

Plant species

Our analysis was based on 95 woody species occurring in temperate and Mediterranean regions of Europe. The species belong to 72 genera and 35 families, with Rosaceae (14 taxa) as the most species-rich family, followed by Betulaceae (8), Salicaceae (7), Fabaceae (6), Fagaceae (6), Oleaceae (6), Ericaceae (5), Anacardiaceae (4) and other families (39). *Prunus* is the most represented genera (5), followed by *Alnus* (4), *Quercus* (4), *Salix* (4), *Populus* (4) and *Acer* (3). Most of the studied taxa are small trees (29), followed by large trees (25), large shrubs (19) and small shrubs (17), and woody lianas (5). Figure 1 shows examples of a few of the species analysed.

Petiole anatomy

For each species, we randomly selected and sampled 3–5 fully expanded and uninjured, sunlit leaves from 3–5 canopy trees. Most samples were obtained during summer field seasons throughout a 5-year period (2012–2016) from trees growing in natural conditions in Switzerland. Six species were sampled in the Arboretum of Průhonice Park, Czech Republic, in summer 2016. Petioles were fixed and stored in 70 % ethanol. The mid-petiole region was selected in order to avoid the anatomical variation that may occur from the leaf base to the insertion point. Cross-sections 20–30 µm thick were made using a sledge microtome. We discoloured the sections with sodium hypochlorite, then double-stained them with a 1 : 1 aqueous Astrablue (0.5 %) and Safranin (1 %) blend, and finally mounted them on a permanent slide with Canada Balsam (Gärtner and Schweingruber, 2013; Schweingruber et al., 2020). The slides were examined using an Olympus BX53 microscope, Olympus DP73 camera and cellSense Entry 1.9 software.

We described a total of 18 anatomical features including cross-sectional geometry (outline), epidermis traits (cell and wall width) and cuticle thickness; the presence of trichomes, hypodermis, collenchyma, fibre band, crystals and secretory structures; and the arrangement of vascular bundles and interfascicular region, phloem (width of parenchyma cells) and xylem (vessel arrangement, vessel diameter and presence of fibres; Fig. 1). A complete characterization of the evaluated traits is presented in the Supplementary Data Figs S1–S50 and Tables S1–S4.

Quantitative anatomical measurements were also performed for each species using the software ImageJ. Cuticle thickness

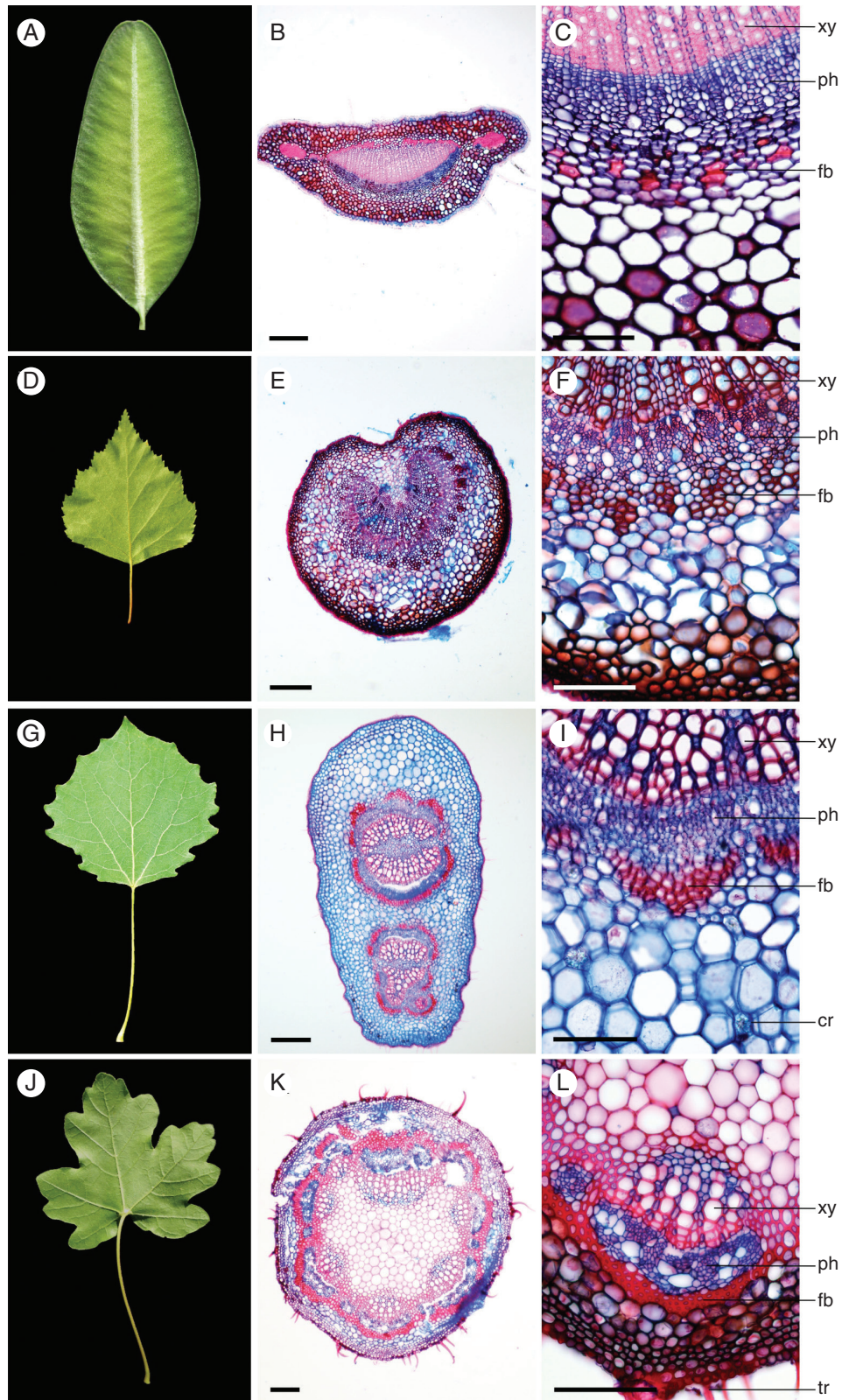


FIG. 1. *Buxus sempervirens* (A–C), *Betula pendula* (D–F), *Populus tremula* (G–I) and *Acer campestre* (J–L). Petiole length is classified in < 2–5 cm (A), 5–10 cm (D, G) and > 10 cm (J). Cross-sections show petiole geometry: horizontally flattened (B), with an indentation (E), vertically flattened (H) and circular (K); and highlight trichomes (tr), fibre band (fb), phloem (ph), xylem (xy) and crystals (cr). Scale bars: 5 cm (A, D, J), 3 cm (G), 200 μ m (B, E, H, K) and 100 μ m (C, F, I, L).

was obtained from an average of 20 measurements made along the periphery of the petiole. Evaluation of tissue predominance was focused on the vascular system (phloem and xylem) and fibre band (sclerenchyma); the total area of each tissue was measured followed by calculating their proportions in relation to the total cross-sectional area of the petiole. For average vessel diameter, we measured all the vessels present in several pre-defined representative 100 × 100 µm squares and average values were then calculated.

Morphology

For most of the species, we obtained data for plant height, leaf dry matter content (LDMC) and specific leaf area (SLA) from the LEDA database (Kleyer *et al.*, 2008). We acquired leaf area (LA) values from the BIEN database (Enquist *et al.*, 2016), TRY database (Kattge *et al.*, 2020) and, in the case of *Cercis siliquastrum*, from the literature (Hatamian *et al.*, 2019). All leaf trait measurements (LA, SLA and LDMC) included whole leaves (i.e. blades and petioles). For compound leaves, values for the whole leaves were used. For six species (i.e. *Alnus cordata*, *Magnolia soulangeana*, *Pyrus communis*, *Rhododendron ferrugineum*, *Ulmus glabra* and *Vitex agnus*) with no data available in databases or the literature, we measured leaf traits in 3–5 sunlit leaves collected from three canopy trees growing in the Arboretum of Průhonice Park, Czech Republic. Leaf area measurements were made using the ImageJ software (Dolezal *et al.*, 2019). Mean values per species were used in further analyses.

Phylogeny

The phylogenetic relationships between the species studied were constructed based on three molecular markers: *matK*, *rbcL* and ITS (internal transcribed spacer). Combined, these three loci cover protein-coding, RNA-coding and non-coding sequences, as well as both plastid (*matK*, *rbcL*) and nuclear DNA (ITS). Varying mutational rates between loci maximizes the ability to discern major lineages together with species-level phylogenies; this threesome of markers has been suggested as a standard for phylogenetic analyses and barcoding of angiosperms (Li *et al.*, 2017).

As a starting point, we acquired all relevant sequences from NCBI GenBank. All three combined matrices (one dataset per locus) were subsequently aligned in MAFFT 6 (Katoh and Toh, 2008), using the L-INS-i algorithm. Partial alignments were concatenated, manually adjusted in BioEdit (Hall, 1999), and subduced to the automated1 algorithm in trimAll software (Capella-Gutiérrez *et al.*, 2009) to exclude highly divergent and gap-rich regions. The best-fit model for phylogenetic inference was selected according to the Bayesian information criterion (Schwarz, 1978) using the Baseml core of the Kakusan4 package (Adachi and Hagesawa, 1996; Tanabe, 2011), resulting in choice of the GTR model with rate variation across locations simulated by a discrete gamma distribution (Γ8), autocorrelated by the AdGamma rates prior, and unlinked for particular gene partitions. This model was subsequently submitted to MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) as the basis for

Markov chain Monte Carlo (MCMC) analysis, encompassing two independent runs with four Metropolis-coupled MCMC chains of 107 generations sampled after every 1000th generation. In each run, one Markov chain was cold and three were incrementally heated by a parameter of 0.3. The first 25 % of entries were discarded as burn-in to eliminate trees sampled before reaching apparent stationarity, and the rest was used to compute the majority-rule consensus.

Data analyses

The estimation of species' climatic preferences was obtained by calculating median values of annual mean temperature (bio1) and annual precipitation (bio12) using bioclimatic variables of the CHELSA climate database (Karger *et al.*, 2016, 2017, 2018) and species coordinates of the GBIF database (Global Biodiversity Information Facility, www.gbif.org; see Supplementary Data Table S5 for DOIs of GBIF occurrence downloads). We used only GBIF coordinates without spatial issues and excluded occurrences that represented absences or were marked as managed. Species occurrences were downloaded from GBIF using the *rgbif* package for R (function *occ_download* with the predicates *hasCoordinate* equals TRUE and *hasGeospatialIssue* equals FALSE). Subsequently, climate data from the CHELSA layers were extracted for each species by the *extract* function of the *raster* package at all coordinates that represented presences (*occurrenceStatus* equals PRESENT) and no intentional cultivation (*establishmentMeans* not equal to MANAGED). To evaluate the effect of species' temperature and precipitation preferences and plant morphology (height, LA, SLA and growth form) on petiole anatomy, we first performed ordination of petiole anatomical features, using non-metric multidimensional scaling, to find major axes of variation in anatomical features and to quantify the variation explained by environment and morphology.

Second, we used the phylogenetic distance-based generalized least squares model (D-PGLS, Adams, 2014b) to quantify how much variation in anatomical settings is explained by LA and other main predictors (plant height, SLA, precipitation and temperature) after accounting for LA while controlling for phylogenetic inertia. Our 18 anatomical variables were used to calculate the distance matrix among all 91 species (four species were excluded due to missing values) using a simple matching coefficient (Legendre and Legendre, 1998):

$$\text{Distance} = 1 - (\text{Number of agreements} / \text{Number of variables})$$

The distance matrix was used as the response; LA, SLA, height, annual precipitation and mean annual temperature were used as predictors. Since the distribution of most predictors was skewed or they had outlier values, the following transformations were performed: natural logarithm of LA, square root of height, and rank values for precipitation and temperature. The implementation of the model we used does not allow simultaneous estimation of the strength of the phylogenetic signal. We estimated and accounted for it with the appropriate degree by running the model with a phylogenetic tree transformed with the varying value of Pagel's lambda (Pagel, 1999). We used lambda values from 0 (no phylogenetic signal) to 1 (phylogenetic

signal corresponding to Brownian motion) varying by 0.01 and selected the model with the highest explained variability. Correlations among predictors were low (strongest being between LA and height, 0.41), and thus explained variability was not highly dependent on the order of the predictors. Therefore, we present explained variability for LA initially, followed by each predictor variable described twice, after accounting only for LA and after accounting for all the other predictors. We also explored the effect of each of our predictors on each anatomical trait separately using the same modelling approach.

Estimation of the phylogenetic signal in the anatomy of petioles alone was performed using the distance matrix that we had constructed for the main model. We also estimated multivariate generalization of Blomberg's *K* (Blomberg et al., 2003; Adams, 2014a) and subsequently employed permutation tests to observe a statistical difference from the Brownian motion model of evolution.

The D-PGLS model was evaluated and the phylogenetic signal was estimated and tested using the package *geomorph* (ver. 3.3.0; Adams et al., 2019), and the phylogenetic tree was scaled using the package *geiger* (ver. 2.0.7; Harmon et al., 2008). All analyses were done in R (ver. 4.0.0; R Core Team, 2020).

RESULTS

In analyses of individual petiole features, LA, plant height, temperature and precipitation were found to be linked to different anatomical traits (Fig. 2). For instance, variation in vessel diameter and vessel arrangement was mainly linked to LA, epidermis cell size to precipitation and plant height, petiole length to LA, cuticle thickness to SLA, and the presence of trichomes to temperature. Epidermal cell wall thickness was mainly related to precipitation. The presence of collenchyma was strongly determined by SLA, and tissue type proportion was linked to precipitation, phloem rays to temperature, and petiole outline to plant height and precipitation.

LA, height, SLA, precipitation and temperature each affected petiole anatomy (*P*-values after accounting for other predictors: 0.028, 0.015, 0.035, 0.006, 0.008, respectively) and together explained 12.19 % of the variability after controlling for phylogeny. The estimated phylogenetic signal of the main model was 1 (Pagel's lambda), which corresponds to the Brownian motion model of evolution. The estimated strength of phylogenetic signal, K_{mult} , in anatomical traits only (without predictors) was 0.3983, which was significantly different from 1 (*P* = 0.001). $K_{mult} < 1$ indicates that the power of the phylogenetic signal

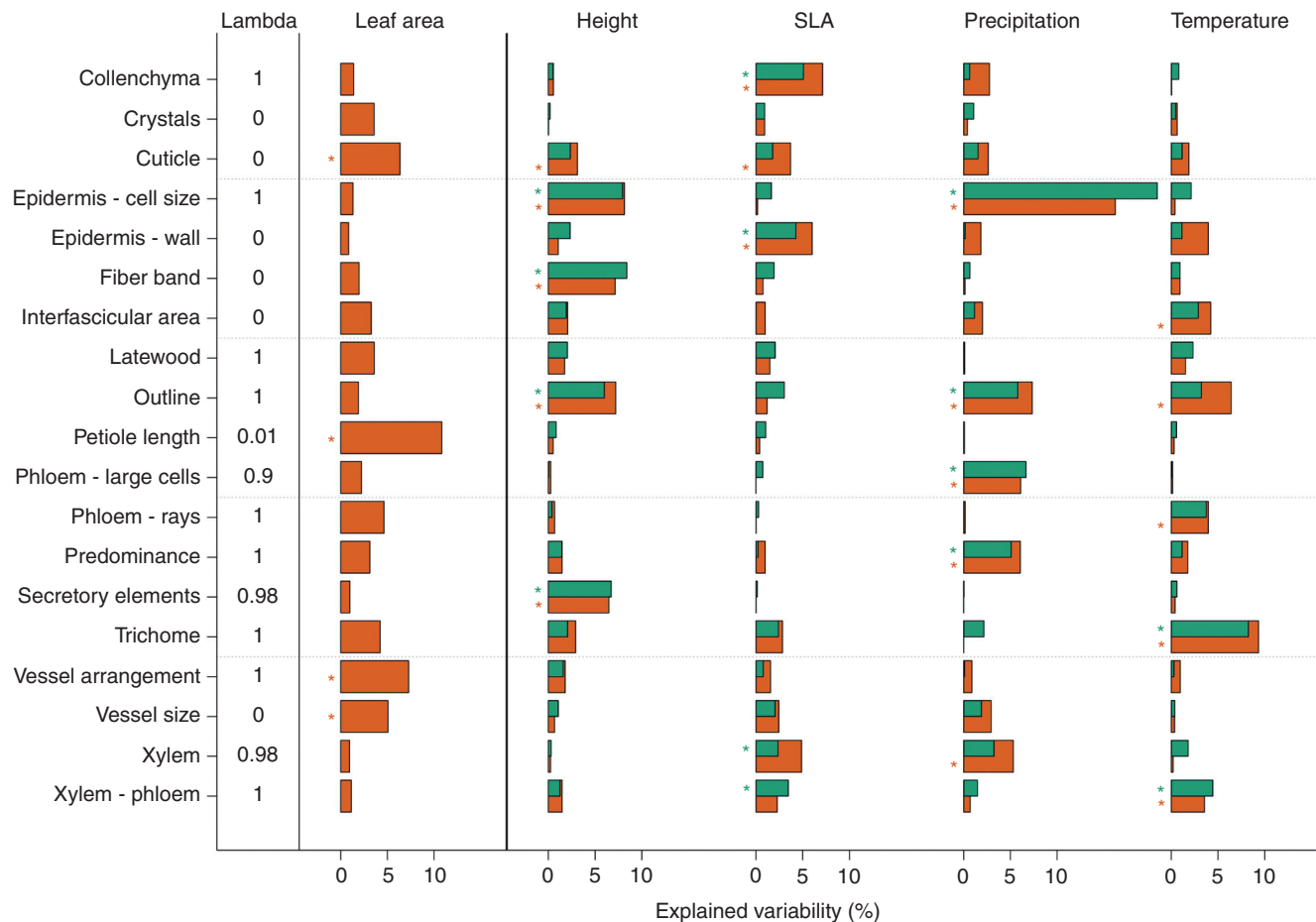


FIG. 2. Explained variability in individual anatomical traits by LA, height, SLA, precipitation and temperature. Brown bars show explained variability accounting only for the covariate LA; green bars denote explained variability after accounting for the other predictors (including LA). **P* < 0.05. In the left column is the estimated strength of the phylogenetic signal in each model (Pagel's lambda). LA = leaf area, SLA = specific leaf area.

was lower than expected under the Brownian motion model of evolution.

NMDS ordination (Fig. 3) showed that the main interspecific differences in petiole anatomy along the first axis were associated with variation in leaf area (LA is positively correlated with SLA), from soft leaves (i.e. composed of more parenchyma tissue and less sclerenchyma) with high SLA to tough leaves (i.e. composed of more sclerenchyma tissue) with low LA and high LDMC (SLA is negatively correlated with LDMC, a result not shown). The petiole anatomical changes along the second

axis were associated with variation across temperature, precipitation and plant height gradients, from cold-adapted shrubs (i.e. families Cornaceae, Grossulariaceae and Aquifoliaceae) to warm-adapted tall trees (i.e. families Sapindaceae and Hippocastaneaceae; Fig. 3).

Trees and shrubs with larger and softer leaves (high LA, high SLA, low LDMC) tended to have longer petioles (Figs 4 and 5) with epidermal cells of the same size as cortex cells, a thin cuticle without trichomes, irregularly arranged wide vessels, a predominance of sclerenchyma, phloem composed of large

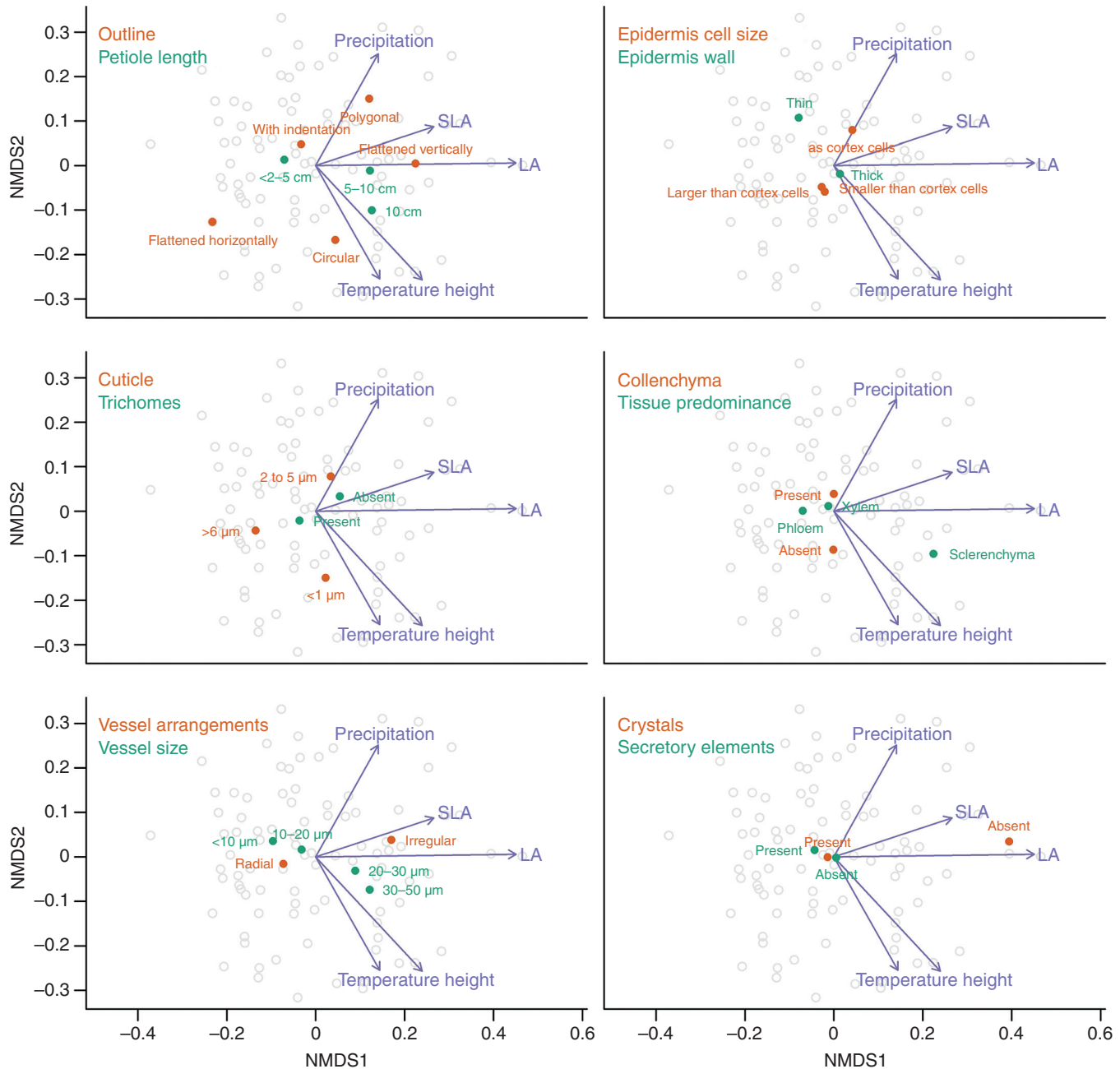


Fig. 3. Ordination of species based on anatomical traits with a projection of predictors and selected anatomical traits. In all six panels, the same ordination is visualized only with projected centroids of levels of different selected traits. NMDS was performed on the distance matrix used in the main model (see Methods), with three axes (only the first two are visualized) and with stress 0.175 [using the package *vegan* (ver. 2.5-6; Oksanen et al., 2018)]. NMDS = non-metric multidimensional scaling, LA = leaf area, SLA = specific leaf area. (See also Supplementary Data Figs S1–S5 for the characterization of the cross-sectional geometry.).

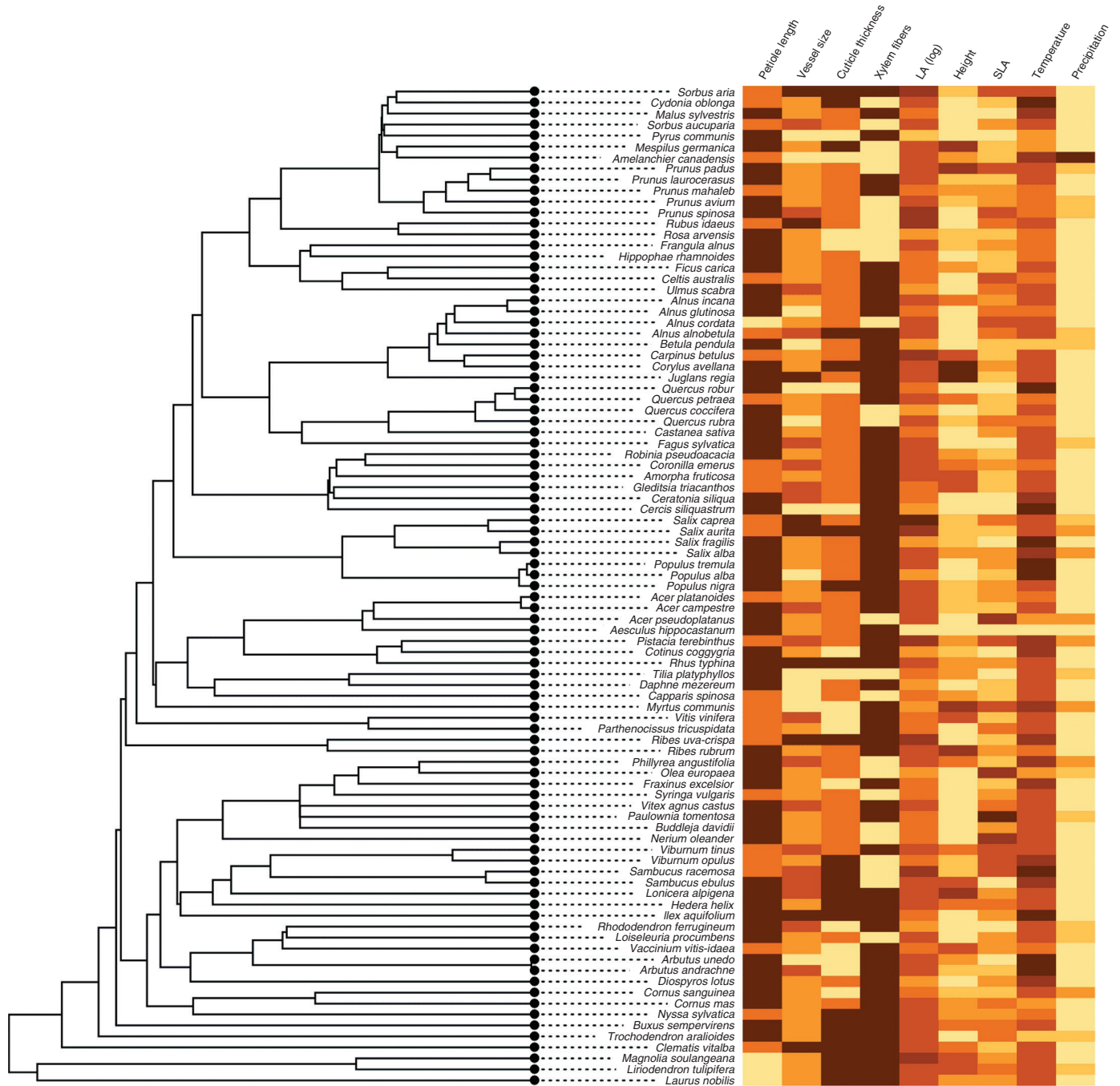


FIG. 4. Phylogenetic tree with visualization of traits and environmental preferences of plants. The lowest values of each trait are in yellow and the highest values in brown.

cells and interfascicular areas with fibres. Petioles of large trees were usually longer (5–10 cm), with almost circular or polygonal external outline, thin cuticle (< 1 μm), xylem fibres present, phloem rays indistinct, and prismatic crystals and druses (Figs 5 and 6). In contrast, trees with smaller leaves (low SLA and high LDMC) were generally smaller statured and from drier habitats with high precipitation seasonality. The petioles of these smaller trees were characterized by a thick cuticle (> 6 μm), simple and non-glandular trichomes, epidermal cells smaller than cortex cells, and phloem composed of small cells and radially arranged vessels (Fig. 3). Moreover, petioles of

smaller trees and shrubs tended to have a circular external form, cuticle width of 2–5 μm, fibreless xylem, fibre band absent, an interfascicular area without fibres and lamellar collenchyma.

Stabilizing elements: petiole outline, epidermis, collenchyma and fibre bands

Plant height and precipitation accounted for the most variation in petiole outline (Fig. 3). A circular outline with flat section or indentation predominated among the studied species

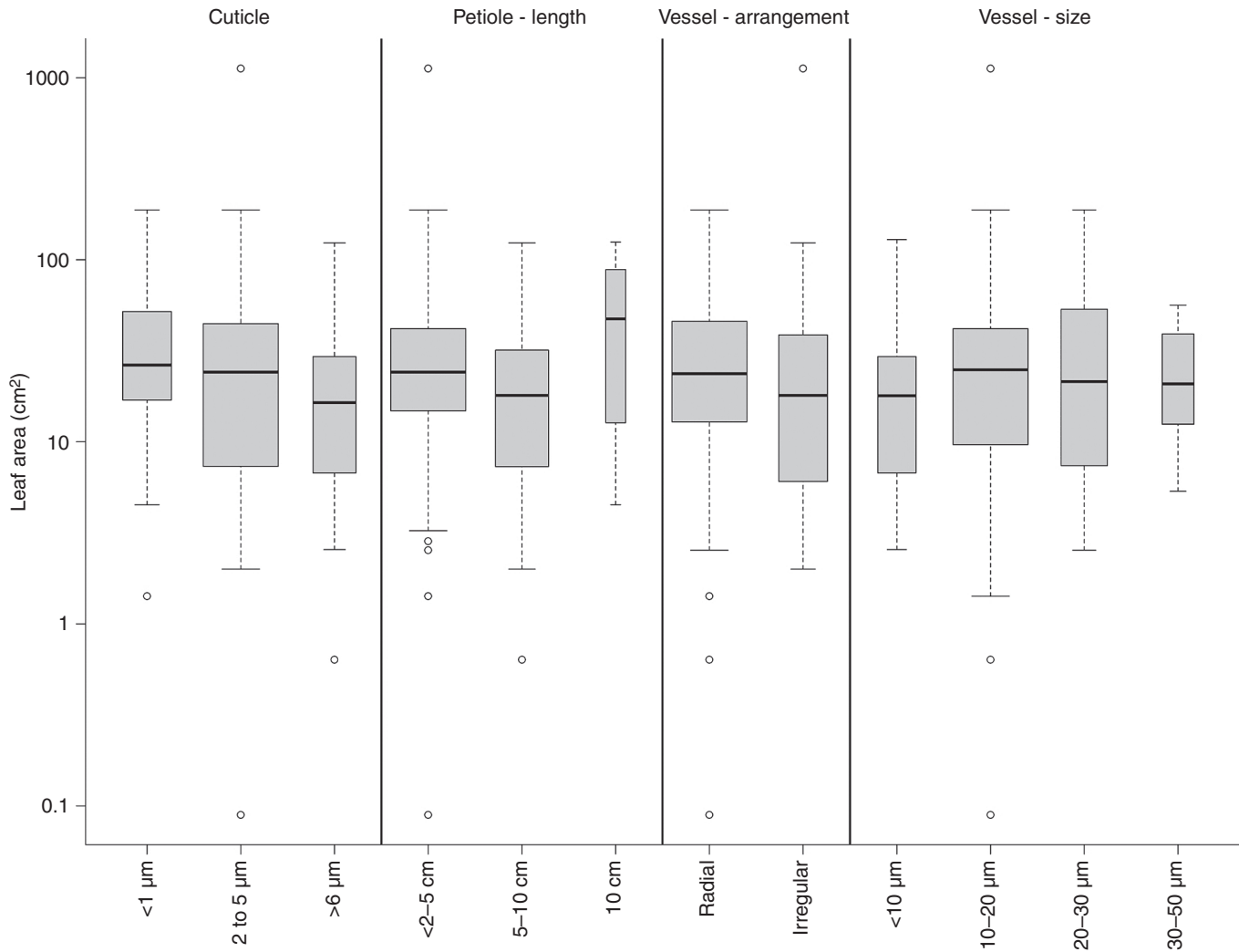


Fig. 5. Relationship between leaf area and selected anatomical traits. The width of boxplots corresponds to the square root of the number of observations in a particular group.

(68 %), typically in shrubs and smaller trees from Cornaceae, Aquifoliaceae, Rosaceae and Oleaceae. A circular petiole outline (22 % of taxa) tended to predominate in regions with lower precipitation (Fig. 3). A vertically flattened outline was found only in *Populus* spp. and horizontally flattened outline in *Buxus sempervirens*. A polygonal outline (5 %) occurred in unrelated taxa such as *Clematis vitalba*, *Loiseleuria procumbens*, *Prunus laurocerasus*, *Ribes rubrum* and *Sambucus ebulus*.

Unlike the petiole outline, the greatest variation in the petiole length was explained by leaf area (Figs 2 and 5). Shorter petioles (<2–5 cm) occurred in 65 % of species, followed by petioles 5–10 cm long (31 %), while longer petioles were found in only 4 % of species including *Acer* spp. and *Ribes rubrum* (Fig. 4). Variation in petiole cuticle thickness was primarily related to LA (Figs 2 and 5). Shrubs and small trees of Cornaceae, Aquifoliaceae, Rosaceae and Oleaceae, accounting for 49 % of all species within the study, had smaller leaves with a thick (2–5 μm) cuticle (Fig. 4). The largest cuticles (>6 μm) were associated with Mediterranean trees such as *Phillyrea angustifolia*, *Quercus coccifera* and *Laurus nobilis* (Fig. 4). Thin cuticles (<1 μm) predominated in large trees with bigger

leaves such as *Acer* spp., *Carpinus betulus*, *Castanea sativa*, *Celtis australis*, *Ceratonia siliqua*, *Cercis siliquastrum* and *Quercus robur* (Supplementary Data Figs S6–S14 for the anatomical characterization of the cuticle).

Plant height and precipitation accounted for the greatest variation in the petiole epidermal cells (Figs 2, 6 and 7). In 56 % of the species, epidermal cells were smaller than cortex cells, usually in smaller trees or shrubs with small SLA such as *Salix*, *Prunus*, *Corylus*, *Vaccinium*, *Viburnum* and *Syringa* (Fig. 4). Tall trees of the genera *Quercus*, *Populus*, *Ulmus* and *Tilia* had petiole epidermal cells of the same size as cortex cells. Petiole epidermal cells were larger than cortex cells in only 6 % of cases, such as in *Aesculus hippocastanum* and *Prunus avium* (see also Supplementary Data Figs S6–S14 for the anatomical characterization of the epidermis). SLA accounted for the greatest variation in epidermal cell-wall thickness (Fig. 8). Most of the tree species with larger SLA had thick-walled epidermal cells (84 %), while thin epidermal cell walls occurred among lianas (*Vitex*, *Hedera*) and shrubs (*Ilex aquifolium*, *Capparis spinosa*, *Viburnum* spp., *Glyziryhza glabra*).

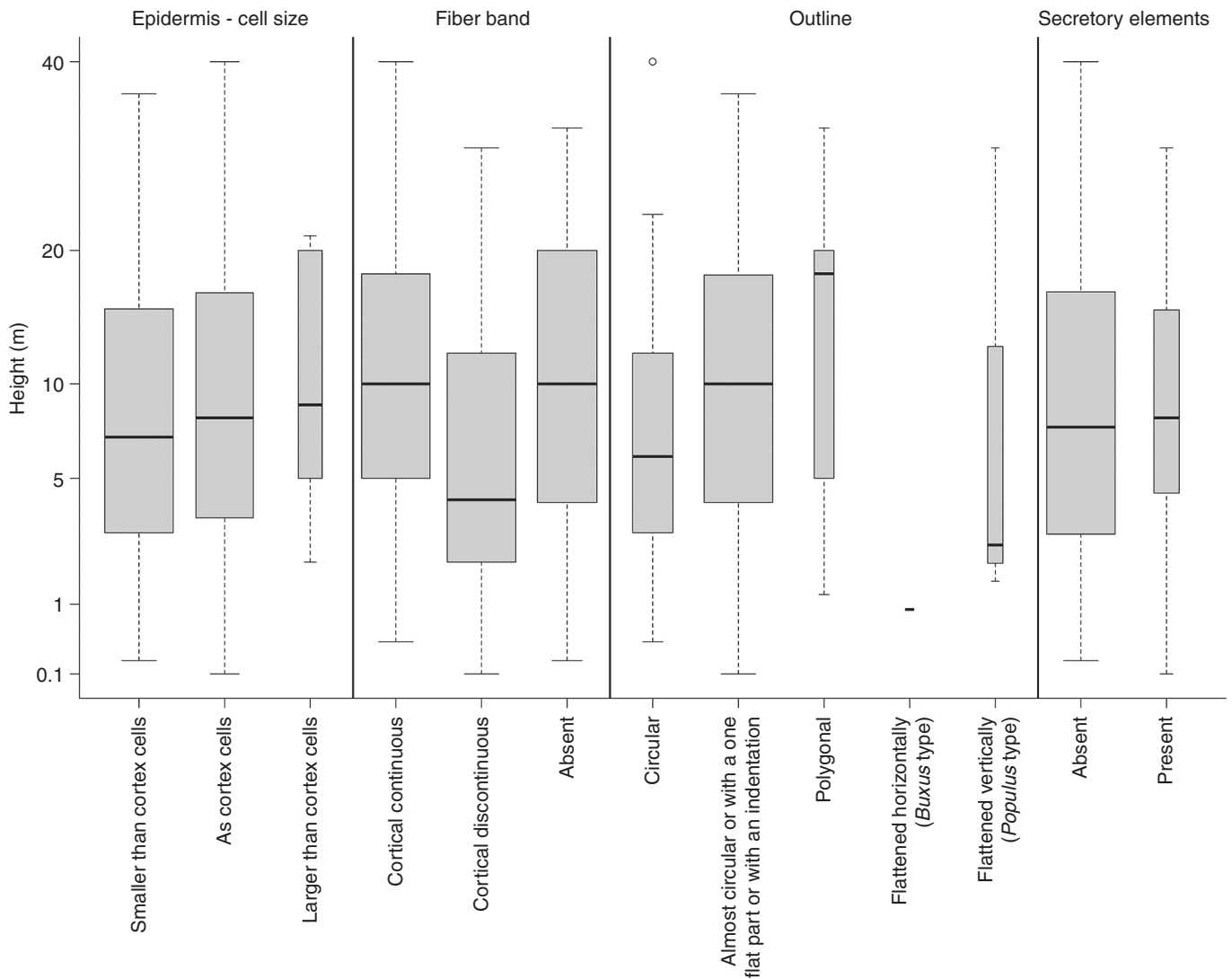


FIG. 6. Relationship between height and selected anatomical traits. The width of boxplots corresponds to the square root of the number of observations in a particular group.

Temperature accounted for the greatest variation in epidermal trichomes (Figs 2 and 9), which are either unicellular (42 %), multicellular (19 %) or absent (39 %). Unicellular and mostly non-glandular trichomes predominated in small trees and shrubs from warmer regions and with high leaf thickness (high LDMC) such as *Buxus sempervirens*, *Cydonia oblonga*, *Nerium oleander*, *Prunus* spp. and *Salix* spp. (Fig. 4). Petiole trichomes were missing in large trees with large and softer leaves such as *Fagus sylvatica*, *Aesculus hippocastanum* and *Robinia pseudoacacia*. Less represented were branched non-glandular trichomes (*Hippophae rhamnoides*, *Olea europaea*, *Quercus coccifera*, *Rhododendron ferrugineum*), simple glandular trichomes (*Corylus avellana*, *Rhus typhina*, *Vitex agnus-castus*) and branched glandular trichomes (*Buddleja davidii*; see also Supplementary Data Figs S6–S14 for the anatomical characterization of the trichomes).

The main mechanical support was achieved through the formation of collenchyma at the periphery of the petioles and

stabilizing cortical fibre bands (see Supplementary Data Figs S15–S21 for the anatomical characterization of the cortex). Variation in collenchyma was mainly related to SLA (Figs 2 and 8), with lamellar collenchyma being found in 70 % of species, mostly in shrubs and small trees with smaller leaves such as from Ericaceae, Cornaceae, Grossulariaceae and Aquifoliaceae, while collenchyma tended to be missing in taller trees with large leaves such as *Populus tremula*, *Paulownia tomentosa* and *Juglans regia* (Fig. 4). Variation in cortical fibre bands was related primarily to plant height (Figs 2, 3 and 6), with fibre bands identified as continuous (36 % of taxa), discontinuous (36 %) or absent (28 %). Fibre bands were largely absent in smaller trees and shrubs from Cornaceae, Aquifoliaceae, Rosaceae, Rhamnaceae, Grossulariaceae and Oleaceae, while continuous cortical fibre bands were highly developed in closely related large trees from Fagaceae (*Quercus*, *Fagus*, *Castanea*), Sapindaceae (*Acer*) and all Fabaceae (*Amorpha fruticosa*, *Ceratonia siliqua*, *Cercis siliquastrum*, *Gleditsia triacanthos*, *Glycyrrhiza glabra* and *Robinia pseudoacacia*; Fig. 4).

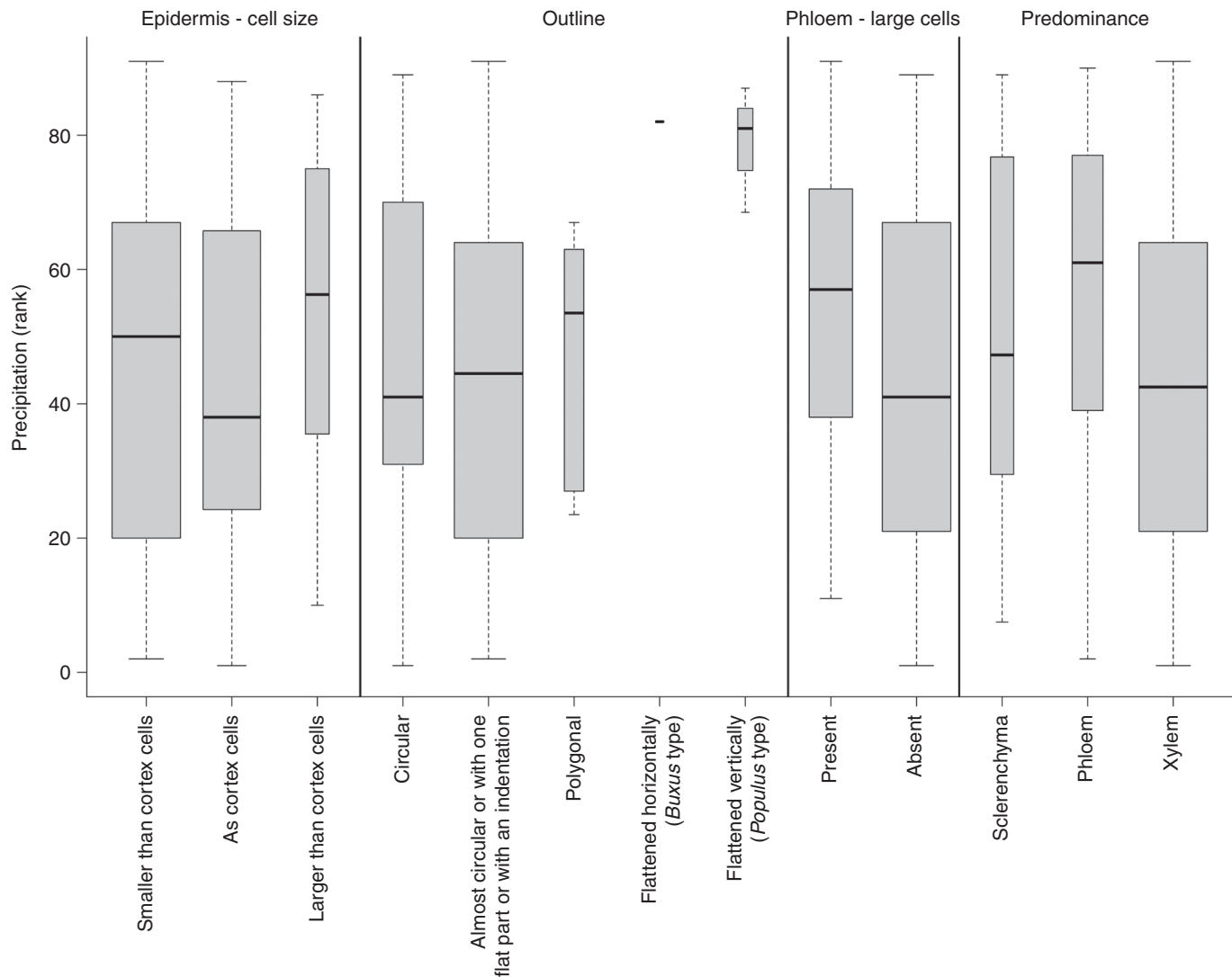


Fig. 7. Relationship between precipitation and selected anatomical traits. The width of boxplots corresponds to the square root of the number of observations in a particular group.

Structure of the conductive system

The structure of vascular bundles was mainly related to SLA and temperature (Figs 3 and 9). Three vascular bundle types were observed among the studied taxa: (1) crescent-shaped phloem and xylem (51 %) were found mostly in shrubs and smaller trees with tough leaves (low SLA), such as *Laurus nobilis*, *Buxus sempervirens*, *Nerium oleander*, *Phillyrea angustifolia*, *Prunus mahaleb* and *Loiseleuria procumbens*; (2) phloem and xylem encircling vascular bundles were found in 41 % of species, mostly large trees with softer leaves (*Quercus*, *Acer*, *Gleditsia*, *Juglans*, *Robinia*); and (3) multiple internal stems were found in (4 %) of the species, *Populus* spp. and *Salix aurita* (see Supplementary Data Figs S22–S33 for the anatomical characterization of the vascular system). In vascular bundles, xylem predominated (76 % of species) over phloem (15 % of species) and sclerenchymatous tissue (9 % of species). Phloem dominating over other tissue types was more frequently found in smaller trees (*Malus*, *Prunus*, *Rhus*, *Salix*) and shrubs (*Lonicera alpigena*, *Rhododendron ferrugineum*),

while sclerenchyma predominated over xylem and phloem in unrelated groups of larger trees from warmer and drier habitats such as in *Liriodendron tulipifera*, *Magnolia × soulangeana*, *Glycyrrhiza glabra* and *Quercus coccifera* (see Figs S34–S48 for the anatomical characterization of the phloem and xylem and tissue proportion). Most of the studied species, including most tall trees, had lignified xylem with fibres composed of small cells (72 %; Fig. 4), while xylem without fibres tended to occur in smaller trees and shrubs across different families but all belonging to Rosopsida (Anacardiaceae, Araliaceae, Aquifoliaceae, Caprifoliaceae, Fabaceae, Rosaceae, Trochodendraceae) (see Figs S34–S41 for the anatomical characterization of the xylem).

The arrangement of vessels in the xylem was best explained by variation in LA (Figs 2 and 3). The vessels were primarily radially distributed among the studied species (71 %) and less frequently irregularly distributed (29 %; Fig. 2). Radially distributed vessels were mostly <20 µm in diameter and typically found in species with smaller and thicker leaves such as

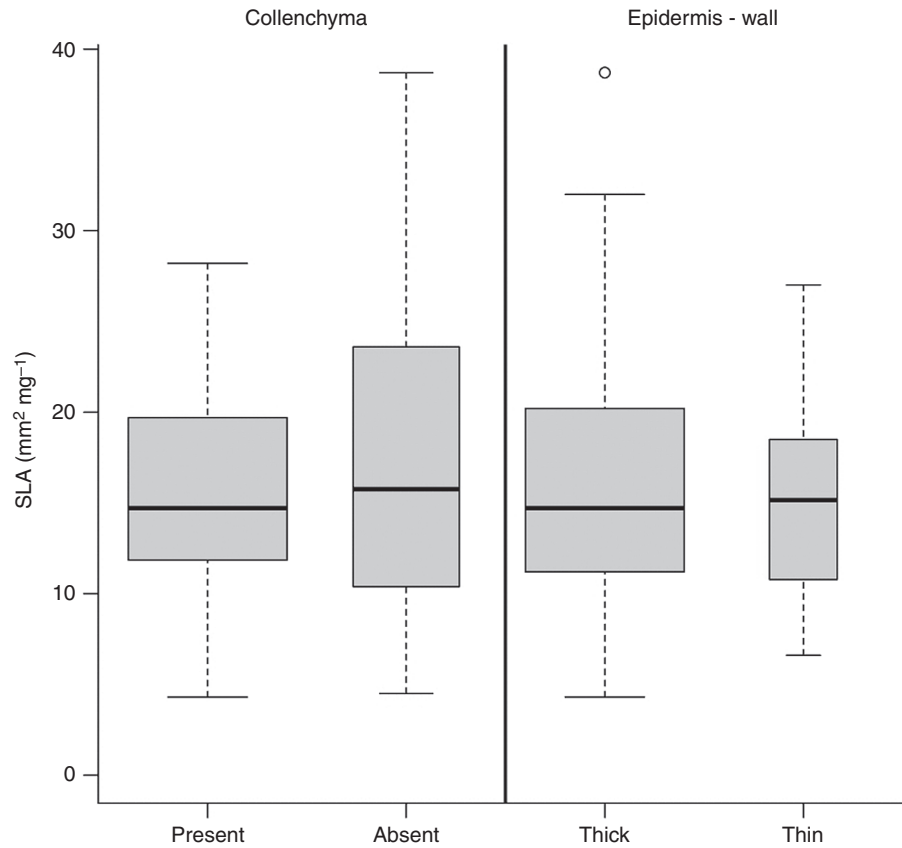


FIG. 8. Relationship between SLA and selected anatomical traits. The width of boxplots corresponds to the square root of the number of observations in a particular group.

Nerium oleander, *Phillyrea angustifolia*, *Buxus sempervirens* and *Arbutus unedo* (see [Supplementary Data Figs S34–S41](#) for the anatomical characterization of the xylem). Irregularly distributed vessels were often $>20\ \mu\text{m}$ in diameter and found in tall trees with softer leaves including *Quercus* and *Populus*, but also lianas such as *Clematis vitalba* and *Hedera helix* (Figs 2 and 4). Variation in vessel diameter was primarily related to leaf size variation (Fig. 5). Narrow vessels ($10\text{--}20\ \mu\text{m}$) occurred in 56 % of taxa, generally in shorter trees and shrubs with smaller leaves, while higher trees with bigger leaves such as *Quercus petraea*, *Juglans regia*, *Aesculus hippocastaneum* and lianas (*Parthenocissus tricuspidata* and *Vitis vinifera*) had wider vessels ($30\text{--}50\ \mu\text{m}$; Fig. 4). The narrowest vessels in petioles were typically found in small shrubs of cold and wet habitats such as *Rhododendron ferrugineum* and *Vaccinium vitis-idaea* or tree species from seasonally dry climates such as *Phillyrea angustifolia*, *Quercus coccifera*, *Ilex aquifolium*, *Gleditsia triacanthos* and *Arbutus unedo* (Fig. 4; see [Figs S42–S48](#) for the anatomical characterization of the xylem vessels). Most of the studied species have phloem composed of small cells (72 %), while the proportion of species with large phloem cells is highest in small trees restricted to a few families such as Rosaceae (*Malus sylvestris*, *Mespilus germanica*, *Pyrus communis*, *Sorbus aria*, *Sorbus aucuparia*), Salicaceae, Sapindaceae and Grossulariaceae. Phloem rays are distinct (49 % of taxa) and indistinct (51 %), influenced by temperature (Fig. 2). The highest share of species with distinct phloem

rays includes smaller trees from Betulaceae, Salicaceae and Rosaceae (Fig. 4).

Crystals and secretory elements

Variation in crystals and secretory elements was primarily related to plant height and LA, respectively (Figs 2 and 3). Distinct crystal types (prismatic, druses, acicular, sand) were found mainly in shorter taxa with smaller and thicker leaves (small SLA and high LDMC, Fig. 3). Species with both prismatic crystals and druses were the most common (46 %), followed by petiole with only druses (26 %), only prismatic crystals (9 %), acicular crystals (6 %), sand (4 %), and druses and sand (2 %). Acicular crystals were found in species from closely related families of the order Lamiales (Lamiaceae, Oleaceae, Paulowniaceae) such as *Olea europaea*, *Syringa vulgaris*, *Paulownia tomentosa* and *Vitex agnus-castus*. Sand crystals were found in *Hippophae rhamnoides*, *Laurus nobilis*, *Phillyrea angustifolia* and *Sambucus* spp. Secretory elements were found only in 12 % of the species. Secretory ducts occurred in 7 % of the species, including small trees (*Myrtus communis*, *Rhus typhina*), shrubs (*Cotinus coggygria*, *Frangula alnus*, *Pistacia lentiscus*), lianas (*Hedera helix*) and large trees (*Tilia platyphyllos*). Slime content was present in 5 % of taxa, exclusively in trees such as *Betula pendula*, *Prunus avium*, *Prunus mahaleb* and *Ulmus glabra*.

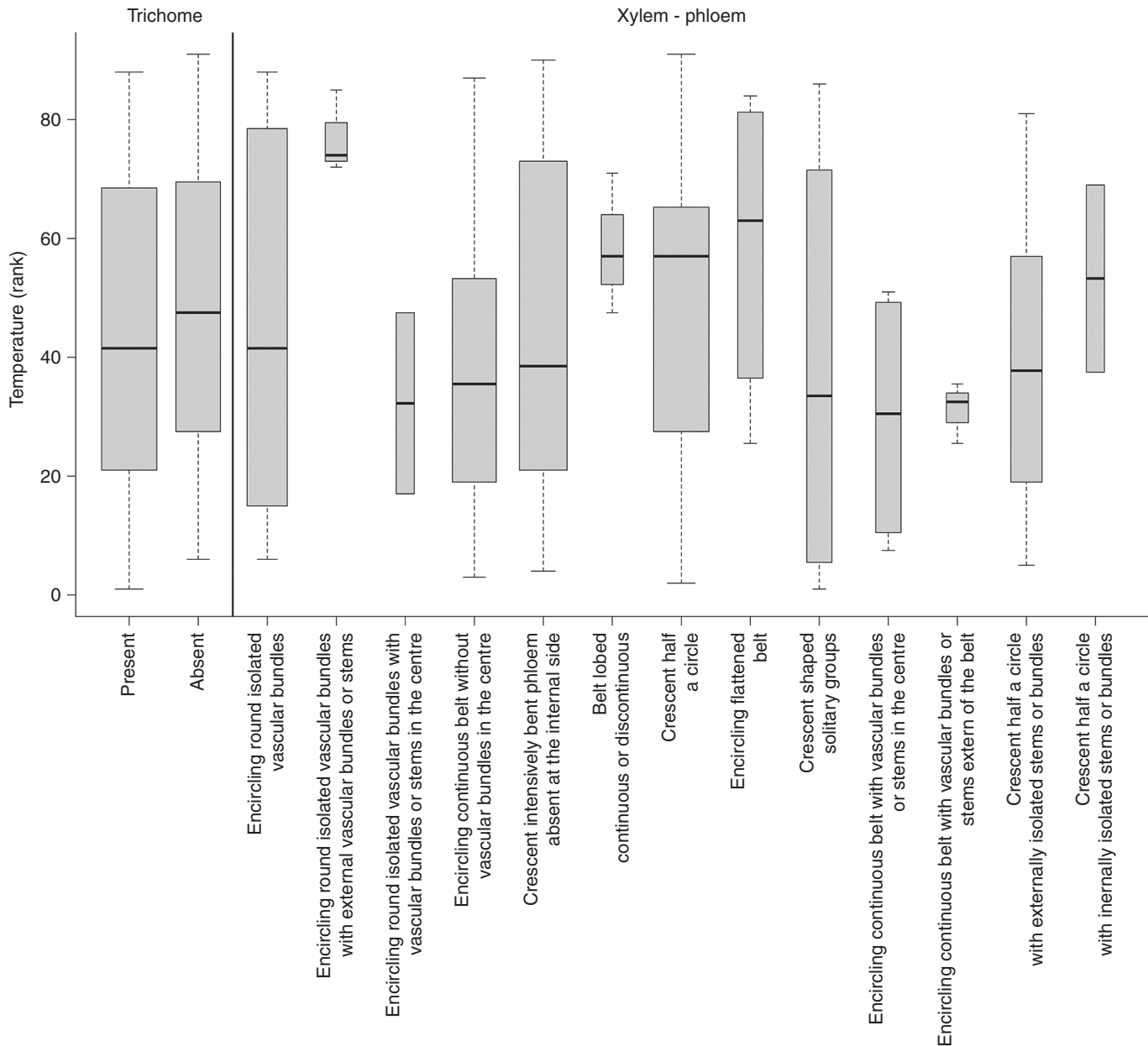


FIG. 9. Relationship between temperature and selected anatomical traits. The width of boxplots corresponds to the square root of the number of observations in a particular group.

DISCUSSION

We present here morphological and anatomical trends in petiole traits of woody species, as a first attempt to provide a broader view on the variation in petioles, which are a key organ supporting the main photosynthetic machinery in leaf blades and playing an essential role in the hydraulic pathway within the plant. Using D-PGLS models, we tested how petiole anatomical features differed according to whole-plant size, leaf traits, thermal and hydrological conditions, and phylogenetic origin in 95 shrubs and trees. We found that species' temperature and precipitation optima, plant height, and leaf area and thickness exerted a significant control on petiole anatomical and morphological structures not confounded by phylogenetic

inertia. Species with different evolutionary histories but similar thermal and hydrological requirements have converged to similar petiole anatomical and hence morphological structures. Our evaluation improves our understanding of how variation in petiole morphoanatomical traits is driven by plant height and leaf characteristics, as well as gradients of temperature and precipitation in an evolutionary context.

Longer petioles tended to be more frequent in larger leaves (higher LA) from taller tree species. The general plant advantage of this tendency is to provide radical changes in leaf orientation and optimize light-harvesting (Takenaka, 1994; Niinemets *et al.*, 2004). Indeed, the combination of petiole length and leaf area directly influences leaf angle, which may reduce the shading of basal foliage and thus increase light-interception

(Niklas, 1992; Takenaka, 1994; Niinemets and Fleck, 2002; Niinemets *et al.*, 2004). The relationship between leaf size (leaf area and petiole length) and plant height also raised questions associated with wind interference. Considering that leaf size may vary with plant height (Niinemets and Kull, 1994), that longer petioles are more flexible to strong winds (Vogel, 1989) and that wind speed increases with height in the canopy (Niinemets and Fleck, 2002), we would expect that longer petioles would be found in leaves of taller trees in order to resist wind bending. Indeed, variation in petiole length is considered to be a leaf strategy to cope with the vertical gradient of wind speed on trees, reinforcing the tendency we observed here.

Additionally to petiole length, petiole cross-sectional geometry (outline) is also an important trait that influences leaf response to wind stresses and leaf self-holding (Niklas, 1992, 1996; Niinemets and Fleck, 2002; Faisal *et al.*, 2012; Louf *et al.*, 2018). In our observations, we identified five different petiole outlines: circular, with indentation (terete petiole), flattened horizontally, flattened vertically and polygonal. We found that those with an indentation and polygonal outline tend to occur in longer petioles from taller trees, while circular outlines tend to be more frequent in shorter petioles from smaller trees. This certainly represents an important morphological trait to understand trends in petiole biomechanics. However, because of the scarce information on this topic, it is difficult to evaluate in detail how outlines influence leaf torsion and bending under wind exposure. Nevertheless, a previous report showed that petioles with a vertically flattened outline, for instance, typical of *Populus* species, promote the reduction of leaf torsion by the wind when compared to petioles with indentation (Niklas, 1992). Our findings call for more experimental studies to better understand how petiole geometry influences leaf flexibility and support among trees.

Despite morphological trends, the main differences between petioles occurred anatomically and were directly influenced by leaf area. In general, larger leaves would require petioles with appropriate anatomical qualities to provide more leaf support and flexibility to handle more intense mechanical stresses (Vincent, 1982; Niklas, 1992). On the first layer of cells, the epidermis width and the thickness of its cell walls are considered to provide important tension-stiffening traits that, together with adjacent tissues, may strongly influence the flexural stiffness of the plant organ (Niklas and Paolillo, 1997; Niinemets and Fleck, 2002). In our observations, smaller leaves tended to present smaller epidermal cells and thin cell walls, which could represent a result of the lower effect of wind when compared to bigger leaves with wider epidermis. Towards the centre of the petiole, the increased frequency of sclerenchyma and collenchyma in larger leaves with longer petioles is consistent with their distinctive biomechanical properties. While collenchyma has soft cell walls, with viscoelastic properties that permit greater petiole bending without breakage, sclerenchyma has lignified and more rigid cell walls, which support and prevent mechanical damage to the adjacent, softer tissues. Indeed, we observed that collenchyma was located on the periphery of petioles, where bending is more intense, while sclerenchyma was associated with vascular bundles, generally located in the centre of the petiole. This tissue organization matches the typical distribution in petioles (Evert, 2006). Moreover, distinct parts of the leaf (midrib, leaf border and leaf apex) characteristically

have well developed collenchyma and sclerenchyma to better hold the mechanical stress rate (Niklas, 1992; Evert, 2006). In the swollen base of petioles, for instance, which holds the leaf and prevents its breakage, the presence of collenchyma is abundant and promotes more plastic movements according to the wind (Niklas, 1992; Evert, 2006), while sclerenchyma is especially located next to the phloem, preventing rupture of the thin cell walls due to mechanical and drought stress (Evert, 2006).

Since leaf anatomy is extremely adaptable to environment conditions (Metcalf and Chalk, 1950; Kröber *et al.*, 2015; Stojnić *et al.*, 2016) and intimately linked to temperature and water gradients (Bussotti *et al.*, 1995; Gravano *et al.*, 1999; Doria *et al.*, 2019), the presence of distinct tissues (epidermis, parenchyma, collenchyma, sclerenchyma) may indicate the conditions to which plants are exposed. However, anatomical variations have mainly described for the leaf blades and less information is available for petioles. We observed that shorter petioles from drier environments, for instance, tend to have smaller epidermal cells and thicker cuticles, which are typical traits to reduce water loss to the environment (Porsch, 1926; Ennajeh *et al.*, 2010; De Micco and Aronne, 2012). Indeed, it is well known that the combination of these anatomical traits together with the reduction of leaf size and the higher proportion of sclerenchyma represent features that are frequently observed in leaves (leaf blade) exposed to water deficiency, high temperatures and light intensity (Castro Díez *et al.*, 1998; Rotondi *et al.*, 2003). However, our findings showed a distinct pattern for sclerenchyma in petioles. We observed that the proportion of sclerenchyma is higher in longer petioles from environments with higher temperatures but with no water deficiency. This tendency may indicate that the main role of sclerenchyma in petioles is to provide biomechanical support instead of preventing water loss, in agreement with the main petiole function of supporting the leaves.

Concerning xylem traits, our analysis showed that vessel diameter is also linked to leaf area, and again with petiole length. While vessels with larger diameter are found in longer petioles (with larger leaves), narrower vessels are developed on shorter petioles (with smaller leaves). Leaf vascular properties are commonly known to be strongly determined by leaf morphology (Salisbury, 1913; Coomes *et al.*, 2008) but consistent responses are also connected to climatic variables (Sellin and Kupper, 2007; Scoffoni *et al.*, 2008; Sack, 2012; Sanginés de Cárcer *et al.*, 2017; Hacke *et al.*, 2017; Kardošová *et al.*, 2020). Even though we observed that xylem vessels tend to enlarge with higher levels of precipitation and temperature, the strongest relationship was still found with leaf size. This tendency agrees with the associations between vessel widening and increasing leaf area previously made in petioles from *Fraxinus americana*, *Quercus robur*, *Acer pseudoplatanus* and other trees (Niklas, 1992; Coomes *et al.*, 2008; Lechthaler *et al.*, 2019; Levionnois *et al.*, 2020). In these studies, the authors analysed single or a few species, but here we show that the same trend occurred when a larger group of species is considered. It is known that longer petioles can only maintain larger leaves with wider vessels (Coomes *et al.*, 2008; Levionnois *et al.*, 2020) as the reduction of vessel diameter would represent a diminished efficiency of water transport to the leaves (Sack *et al.*, 2003; Brodribb, 2009; Jordan *et al.*, 2013; Scoffoni *et al.*, 2016; Levionnois *et al.*, 2020). This is in line with previous observations of

vessels widening within the leaf, from the very narrow vessels at the end of the sap path close to the stomata towards the wider vessels at the base of the leaf (Rosell *et al.*, 2017, 2019). However, a decrease in vessel diameter in colder, drier climates provides a safer hydraulic structure, less vulnerable to embolisms induced by drought or freezing conditions, as narrow vessels are more resistant to cavitation (Zimmermann, 1983; Cochard, 2002).

Another interesting anatomical trend revealed here is the presence of different cell types in vascular tissues, which may be considered relevant for the taxonomy of certain groups. Despite the high plasticity of leaf anatomy, different studies have shown that vascular patterns and superficial traits (epidermis, cuticle, trichomes) in petioles have an important value for the taxonomy of distinct groups (Hare, 1944; Kocsis and Borhidi, 2003; Noraini *et al.*, 2016; Talip *et al.*, 2017; Anu and Dan, 2020; Karaismailoğlu, 2020). Our findings showed that in small trees and shrubs from some families (Anacardiaceae, Araliaceae, Aquifoliaceae, Caprifoliaceae, Fabaceae, Grossulariaceae, Rosaceae, Salicaceae, Sapindaceae, Trochodendraceae) the phloem has scattered larger parenchymatic cells and the xylem is fibreless. Even though neither of these traits has been considered to be axonomically relevant in petioles, in woody stems the patterns of parenchyma and the presence and absence of fibres are defined as the most conspicuous traits to characterize species (Chattaway, 1953; Roth, 1981; Archer and van Wyk, 1993; den Outer, 1993). Moreover, previous studies showed similar characteristics on the midrib of the same species (Săvulescu and Luchian, 2009; El-Alfy *et al.*, 2011; Koçyiğit *et al.*, 2015; Jušković *et al.*, 2017). Even though anatomical changes occur along the petiole and at the beginning of the midrib (Sack and Holbrook, 2006), both traits seem to have the potential for taxonomic applications.

The structural traits of petioles we evaluated in this study included morphological and anatomical aspects that characterized their shape, stiffness and hydraulic potential. Our analysis was based on 95 major woody species from Europe representing one of the first attempts to find trends for the broad and diversified group of woody plants. Our results showed that leaf area has the strongest influence on petiole anatomical traits, more than temperature, precipitation and plant height, emphasizing the supportive and mechanical role of this part of the leaf. Petioles tend to be longer and have a circular outline in larger leaves. Anatomically, mechanically supportive cells (colenchyma and sclerenchyma) tend to be more predominant and xylem vessels tend to have a larger diameter in bigger leaves. These traits are aligned with our expectations, as bigger leaves represent more weight for self-holding and demand a more efficient vascular tissue. In the case of smaller leaves, petioles tend to be shorter, to have an outline with an indentation and have narrower vessels. Indeed, environmental factors may directly influence distinct plant organs, but our results appear to reveal a different pattern for petioles that relates to the leaf itself. Since this study is constrained geographically to Europe and hence is based on a limited set of woody species selected from otherwise highly species-rich functional groups of trees and shrubs, further investigations are needed to better understand the evolution of petiole structures in temperate and especially tropical woody species.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Figures S1–S5: Characterization of the cross-sectional geometry. Figures S6–S14: Anatomical characterization of the epidermis. Figures S15–S21: Anatomical characterization of the cortex. Figures S22–S33: Anatomical characterization of the vascular system. Figures S34–S41: Anatomical characterization of the phloem and xylem. Figures S42–S48: Anatomical characterization of the xylem vessels and tissue proportion. Figure S49: Variability of petiole anatomy by LA, height, SLA, temperature and precipitation. Figure S50: Phylogenetic tree of species' morphoanatomical variation. Table S1: List of anatomical features described for cross-sectional geometry and epidermis of petioles from each species. Table S2: List of anatomical features described for the cortical area and vascular bundles of petioles from each species. Table S3: List of anatomical features detailing vascular tissues in each species. Table S4: List of anatomical features described for tissue predominance and presence and absence of crystals and secretory structure from each species. Table S5: List of GBIF download DOIs for each species and climatic preferences of temperature and precipitation.

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