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## Comparative anatomy of leaf petioles in temperate trees and shrubs: the

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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 about their structural adaptations across evolutionary lineages and environmental conditions. To fill our knowledge gap, we investigated the variation of petiole morphology and anatomy of mainly European woody species to better understand drivers of internal and external constraints in an evolutionary context.

Methods We studied how petiole anatomical features differed according to wholeplant size, leaf traits, thermal and hydrological conditions, and taxonomical 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 anatomically characterized by the predominance of sclerenchyma, larger vessels, interfascicular areas with fibers 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 terete outline, phloem composed of small cells and radially arranged vessels, fiberless xylem and lamellar collenchyma. Individual anatomical traits were linked to different internal and external drivers. The petiole length and vessel diameter increase with enlarging 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, 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.

Keywords: 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 leaf blade size variation (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 few closely related species (Niklas, 1991; Aasamaa and Sõber, 2010; Brocious and Hacke, 2016; Mahley at al., 2018), which provide a narrow view of potential variation. Petiole anatomical studies are mainly descriptive, focusing on the characterization of distinct taxa (Metcalfe 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 (Nicklas, 1991, 1992; Tadrist 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 being compared with the size of 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 Sõber, 2008; 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 petioles morphoanatomy of one or a few species with abiotic conditions such as desiccation, light availability and wind gradient are available (Nicklas, 1991; 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 largestatured and large-leaved plants predominate in wet, warm and productive environments, while short-statured and small-leaved plants occur in cold or arid conditions, such as 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 diameterreflects environmental constraints operating at the whole-plant level (Hacke et al., 2016), while the other relates increasing vessel diameter along the plant stem to accomplish sap conduction requirements (Olson et al., 2013). However, to our
knowledge, there are no studies until now that would combine 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, we investigated the variation of petiole morphology and anatomy among a census of mainly European woody species growing in contrasting environmental conditions using phylogenetic comparative models (Adams et al., 2014). In particular, we studied how petiole anatomical features differed according to whole-plant size, leaf traits, thermal and hydrological conditions, and taxonomical 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, 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., 2015), we expected the vessel diameter to increase with the 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 few species that we analyzed in this work.

## 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 five year period (2012-2016) from trees growing in natural conditions in Switzerland. Six species were sampled in the Arboretum of the Průhonice Park, Czech Republic in summer 2016. Petioles were fixated 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 $\mu \mathrm{m}$ thick were made using a sledge microtome. We discolored the sections with sodium hypochlorite, then double-stained them with a $1: 1$ aqueous Astrablue (0,5\%) and Safranin (1\%) blend, and finally mounted 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), cuticle thickness; the presence of trichomes, hypodermis, collenchyma, fiber band, crystals and secretory structures; the arrangement of vascular bundles and interfascicular region, phloem (width of parenchyma cells) and xylem (vessel arrangement, vessel diameter and presence of fibers; Figure 1). The complete characterization of the evaluated traits is presented in the Supplementary data figures S1-S50 and Supplementary data tables S1-S4.

Quantitative anatomical measurements were also performed for each species using the software ImageJ. Cuticle thickness was obtained with an average of 20 measurements made along the periphery of the petiole. The evaluation of tissue predominance was focused on the vascular system (phloem and xylem) and fiber band (sclerenchyma); the total area of each tissue was measured followed by calculating their proportions in relation to the total crosssectional area of the petiole. For the average vessel diameter, we measured all the vessels present in several pre-defined representative squared areas with $100 \mu \mathrm{~m}$ on each side and average values were then calculated.

## Morphology

For most of the species, we obtained data of plant height, leaf dry matter content (LDMC), specific leaf area (SLA) from LEDA database (Kleyer et al., 2008). We acquired leaf area (LA) values from 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, Ulnus glabra and Vitex agnus) with no data available in databases or litarature, we measured leaf traits in 3-5 sunlit leaves collected from 3 canopy trees growing in the Arboretum of the Průhonice Park, Czech Republic. Leaf area measurements were done 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. Combined, these three loci cover proteincoding, RNA-coding, noncoding 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 was therefore suggested as a standard for phylogenetic analyses and barcoding of angiosperms in the past (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 afterward 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 subdued 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 Kakusan4 package (Adachi and Hagesawa, 1996; Tanabe, 2011), resulting in the choice of the GTR model with rate variation across locations simulated by a discrete gamma distribution (Г8), autocorrelated by the AdGamma rates prior, and unlinked for particular gene partitions. This model was afterward submitted to MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) as the basis for 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 downloadsWVe 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 was extracted for each species by the extract function of the raster package at all coordinates which 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 nonmetric multidimensional scaling, to find major axes of variation in anatomical features and to quantify the variation explained by environment and morphology.

Secondly, we used the phylogenetic distance-based generalized least squares model (D PGLS, Adams, 2014 b) 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 ( 4 species excluded due to missing values) using a simple matching coefficient (Legendre and Legendre, 1998):

Distance $=1$ - (Number of agreements $/$ 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 leaf area, square root of height, and rank values for precipitation and temperature. The used implementation of the model 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) by 0.01 and selected the model with the highest explained variability. Correlations among predictors were low (strongest being between leaf area and height 0.41 ), thus explained variability was not highly dependent on the order of the predictors. Therefore, we present explained variability for leaf area initially, followed by each predictor variable described twice, after accounting only for leaf area 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 which we had constructed for the main model. We also estimated multivariate generalization of Blomberg's K (Blomberg et al., 2003; Adams, 2014 a) 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 package geomorph (version 3.3.0; Adams et al., 2019), phylogenetic tree was scaled using package geiger (version 2.0.7; Harmon et al., 2008). All analyses were done in R (version 4.0.0; R Core Team, 2020).

## RESULTS

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

Leaf area, height, SLA, precipitation, and temperature each affected petiole anatomy (p-values after accounting for other predictors: $0.028,0.015,0.035,0.006,0.008$ ) and explained together $12.19 \%$ 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-value 0.001). Kmult lower than 1 means the power of phylogenetic signal lower than expected under the Brownian motion model of evolution.

NMDS ordination (Figure 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 by more parenchyma tissue and less schlerenchyma) with high SLA to tough leaves (i. e. composed by more schlerenchyma 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; Figure 3).

Trees and shrubs with bigger and softer leaves (high LA, SLA, low LDMC) tended to have longer petioles (Figures 4 and 5) with epidermal cells of the same size as cortex cells, a thin cuticle without trichomes, irregularly arranged wide vessels, the predominance of sclerenchyma, phloem composed of large cells, and interfascicular areas with fibers. Petioles of large trees were usually longer ( $5-10 \mathrm{~cm}$ ), with almost circular or polygonal external outline, thin cuticle $<1 \mu \mathrm{~m}$, xylem fibers present, phloem rays indistinct, and prismatic crystals and druses (Figures 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 $>6 \mu \mathrm{~m}$, simple and non-glandular trichomes, epidermal cells smaller than cortex cells, and phloem composed of small cells and radially arranged vessels (Figure 3). Moreover, petioles of smaller trees and shrubs tended to have a circular external form, cuticle width of 2 to $5 \mu \mathrm{~m}$, fiberless xylem, fiber band absent, an interfascicular area without fibers, and lamellar collenchyma.

Stabilizing elements: petiole outline, epidermis, collenchyma, and fiber bands Plant height and precipitation accounted for the most variation in the petiole outline (Figure 3). The circular outline with flat section or indentation predominated among the studied species (68\%), typically in shrubs and smaller trees from Cornaceae, Aquifoliaceae, Rosaceae, and Oleaceae. Circular petiole outline ( $22 \%$ of taxa) tended to predominate in regions with lower precipitation (Figure 3). The vertically flattened outline was found only in Populus spp. and horizontally flattened outline in Buxus sempervirens. 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 most variation in the petiole length was explained by the leaf area (Figures 2 and 5). Shorter petioles ( $<2-5 \mathrm{~cm}$ ) occurred in $65 \%$ of species, followed by 5-10 cm long petioles (31\%), while longer petioles were found in only $4 \%$ of species including Acer spp. and Ribes rubrum (Figure 4). Variation in petiole cuticle thickness was primarily related to LA (Figures 2 and 5). Shrubs and small trees of the Cornaceae, Aquifoliaceae, Rosaceae, and Oleaceae, accounting for $49 \%$ of all species within the study, were found to have smaller leaves with a thick, 2 to $5 \mu \mathrm{~m}$ cuticle (Figure 4). The largest cuticles (> $6 \mu \mathrm{~m}$ ) were associated with Mediterranean trees such as Phillyrea angustifolia, Quercus coccifera and Laurus nobilis (Figure 4). Thin cuticles (< $1 \mu \mathrm{~m}$ ) predominated in large trees with bigger leaves such as Acer spp, Carpinus betulus, Castanea sativa, Celtis australis, Ceratonia siliqua, Cercis siliquastrum, and Quercus robur (see Supplementary data figures S6-S14 for the anatomical characterization of the cuticle).

Plant height and precipitation accounted for the most variation in the petiole epidermal cells (Figures 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 (Figure 4). Tall trees of 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 figures S6-S14 for the anatomical characterization of the epidermis). SLA accounted for the most variation in the epidermal cell-wall thickness (Figure 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., Glyzirhyza glabra).

Temperature accounted for the most variation in epidermal trichomes (Figures 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 high leaf thickness (high LDMC) such as Buxus sempervirens, Cydonia oblonga, Nerium oleander, Prunus spp, and Salix spp (Figure 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 figures 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 fiber bands (see Supplementary data figures S15-S21 for the anatomical characterization of the cortex). Variation in collenchyma was mainly related to SLA (Figures 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 (Figure 4). Variation in cortical fiber bands was primarily related to plant height (Figures 2-3 and 6), with fiber bands identified as continuous (36\% of taxa), discontinuous (36\%), or absent (28\%). Fiber bands were largely absent in smaller trees and shrubs from Cornaceae, Aquifoliaceae, Rosaceae, Rhamnaceae, Grossulariaceae, and Oleaceae families, while continuous cortical fiber 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, Figure 4).

## Structure of the conductive system

The structure of vascular bundles was mainly related to SLA and temperature (Figures 3 and 9). Three categorical vascular bundle types were observed among the studied taxa: i) crescent-shaped phloem and xylem (51\%) was 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; ii) phloem and xylem encircling vascular bundles were found in $41 \%$ of species, mostly large trees with softer leaves (Quercus, Acer, Gleditsia, Juglans, Robinia); and iii) multiple internal stems were found in (4\%) of the species, Populus spp and Salix aurita (see Supplementary data figures 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 predominates over xylem and phloem in larger trees from warmer and drier habitats unrelated groups such as in Liriodendron tulipifera, Magnolia x soulangeana, Glycyrrhiza glabra and Quercus coccifera (see Supplementary data figures 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 fibers composed of small cells ( $72 \%$, Figure 4), while xylem without fibers tended to occur in smaller trees and shrubs across different families but all belonging to Rosopsida (Anacardiaceae, Araliaceae, Aquifoliaceae, Caprifoliaceae, Fabaceae, Rosaceae,

Trochodendraceae) (see Supplementary data figures S34-S41 for the anatomical characterization of the xylem).

The arrangement of vessels in the xylem was best explained by variation in LA (Figures 2 and 3). The vessels are primarily radially distributed among studied species (71\%) and less frequently irregularly distributed (29\%; figure 2). Radially distributed vessels were mostly smaller than $20 \mu \mathrm{~m}$ in diameter and typically found in species with smaller and thicker leaves such as Nerium oleander, Phillyrea angustifolia, Buxus sempervirens, Arbutus unedo (see Supplementary data figures S34-S41for the anatomical characterization of the xylem), Irregularly distributed vessels were often larger than $20 \mu \mathrm{~m}$ in diameter and are found in tall trees with softer leaves including Quercus and Populus, but also lianas such as Clematis vitalba and Hedera helix (Figures 2 and 4). Variation in vessel diameter was primarily related to leaf size variation (Figure 5). Narrow vessels (10-20 $\mu \mathrm{m}$ ) 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) have wider vessels (30-50 $\mu \mathrm{m}$, Figure 4). The narrowest vessels in petioles are 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 (Figure 4; see Supplementary data figures 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 the highest in small trees restricted to few families like 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 (Figure 2). The highest share of species with distinct phloem rays includes smaller trees from Betulaceae, Salicaceae, and Rosaceae families (Figure 4).

## Crystals and Secretory elements

Variation in crystals and secretory elements was primarily related to plant height and leaf area, respectively (Figures, 2-3). Distinct crystal types (prismatic, druses, acicular, sand) were found mainly in shorter taxa with smaller and thicker leaves (small SLA and high LDMC, Figure 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 are found in species from closely related families from order Lamiales (Lamiaceae, Oleaceae, Paulowniaceae) such as Olea europaea, Syringa vulgaris, Paulownia tomentosa and Vitex agnus-castus. Sand crystals are found in Hippophae rhamnoides, Laurus nobilis, Phillyrea angustifolia and Sambucus spp. Secretory elements were found only in $12 \%$ of the species. Secretory ducts occur 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 is present in $5 \%$ of taxa, exclusively in trees such as Betula pendula, Prunus avium and P. mahaleb and Ulmus glabra.

## DISCUSSION

We presented 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 on the hydraulic pathway within the plant. Using phylogenetic distance-based generalized least squares 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, 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 the plant height and leaf characteristics, as well as gradients of temperature and precipitation in 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 increasing light-interception (Niklas, 1991; Takenaka, 1994; Niinemets and Fleck, 2002; Niinemets et al., 2004). The relation 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 the wind speed increase 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, 1991, 1996; Niinemets and Fleck, 2002; Faisal et al., 2012; Louf et al., 2018). In our observations, we identified five different petiole outlines, including circular, with indentation
(terete petiole), flattened horizontally and vertically, and polygonal. We found that petioles 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 fact 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 the way 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, 1991). Our findings call for more experimental studies to better understand how petiole geometry influences leave 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, 1990). 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 the wind when compared to bigger leaves with wider epidermis. Towards the center 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
damages 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 center of the petiole. This tissue organization is in alignment with the typical distribution in petioles (Evert, 2006). Moreover, distinct parts of the leaf (midrib, leaf border and leaf apex) are characterized to have both tissues more well developed to better hold mechanical stress rate (Niklas, 1991; Evert, 2006). In the swollen base of petioles, for instance, which holds the leaf and prevent its breakage, the presence of collenchyma is abundant and promote more plastic movements according to the wind (Niklas, 1991; Evert, 2006), while sclerenchyma is especially located next to the phloem, preventing the rupture of the thin cell walls due to mechanical and drought stress (Esau, 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 with temperature and water gradients (Bussotti et al., 1995; Gravano et al., 1999; Doria et al., 2019), the presence of distinct tissues (epidermis, parenchyma, colenchyma, schlerenchyma) may indicate the conditions to which plants are exposed. However, anatomical variations are 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 schlerenchyma represent features that are frequently observed in leaves (leaf blade) exposed to water deficiency, high temperatures and light intensity (Castro Diez et al., 1997; 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, which is alignned with the main petiole function of supporting the leaves.

Concerning xylem traits, our analysis showed that vessel diameter is also linked with leaf area, and again with petiole length. While vessels with bigger 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 with 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 is aligned with the associations between vessel widening and increasing leaf area previously made in petioles from Fraxinus americana, Quercus robur, Acer pseudoplatanus and other trees (Nicklas, 1992; Coomes et al., 2008; Lechthaler et al., 2019; Levionoides et al., 2020). In these studies, the authors analyzed single or 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; Levionoides et al., 2020) as the reduction of vessel diameter would represent diminished efficiency of water transport to the leaves (Sack et al., 2003; Brodribb, 2009; Jordan et al., 2013; Scoffoni et al., 2016; Levionoides 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; Rosell et al., 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 resistence to cavitation (Zimmermann, 1983; Cochard, 2002).

Another interesting anatomical trend showed 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 proven 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, 2020Karaismailoğlu, 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 bigger parenchymatic cells and the xylem is fiberless. Even though both traits have not been yet considered taxonomically relevant in petioles, in woody stems the patterns of parenchyma and the presence and absence of fibers are defined as the most conspicuous traits to characterize species (Chattaway, 1953; Roth, 1981; Archer and van Wyk, 1993; den Outer, 1993). Moreover, preview 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, which emphasize 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 (collenchyma and sclerenchyma) tend to be more predominant and xylem vessels tend to have a bigger 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 indentation and narrower vessels. Indeed, environmental factors may directly influence distinct plant organs, however, 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 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.

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## FIGURE CAPTIONS

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

Figure 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). Stars show the cases with a p-value below 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.

Figure 3. Ordination of species based on anatomical traits with a projection of predictors and selected anatomical traits. In all 6 panels, the same ordination is visualized only with projected centroids of levels of different selected traits. NMDS was done on distance matrix used in the main model (see methods), with 3 axes (only the first 2 are visualized) and with stress 0.175 (using package vegan (version 2.5-6; Oksanen et al. 2018)). NMDS = non-metric multidimensional scaling, LA = leaf area, SLA = specific leaf area. (See also Supplementary data figures S1-S5 for the characterization of the cross-sectional geometry).

Figure 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.

Figure 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.

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

Figure 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.

Figure 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.

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

Figure 1


Figure 2


Figure 3


Figure 4


Figure 5


Figure 6


Figure 7


Figure 8


Figure 9


