Deciduous and evergreen oaks show contrasting adaptive responses in leaf mass per area across environments

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Summary

- Increases in leaf mass per area (LMA) are commonly observed in response to environmental stresses and are achieved through increases in leaf thickness and/or leaf density. Here, we investigated how the two underlying components of LMA differ in relation to species native climates and phylogeny, across deciduous and evergreen species.
- Using a phylogenetic approach, we quantified anatomical, compositional and climatic variables from 40 deciduous and 45 evergreen *Quercus* species from across the Northern Hemisphere growing in a common garden.
- Deciduous species from shorter growing seasons tended to have leaves with lower LMA and leaf thickness than those from longer growing seasons, while the opposite pattern was found for evergreens. For both habits, LMA and thickness increased in arid environments. However, this shift was associated with increased leaf density in evergreens but reduced density in deciduous species.
- Deciduous and evergreen oaks showed fundamental leaf morphological differences that revealed a diverse adaptive response. While LMA in deciduous species may have diversified in tight coordination with thickness mainly modulated by aridity, diversification of LMA within evergreens appears to be dependent on the infrageneric group, with diversification in leaf thickness modulated by both aridity and cold, while diversification in leaf density is only modulated by aridity.

Introduction

Sclerophyllous leaves are tough and frequently hard and stiff (Grubb, 1986; Turner, 1994; Read & Sanson, 2003). To obtain a quantitative proxy value for this leaf trait, most ecophysiological studies calculate either the specific leaf area or its inverse, the leaf mass per unit area (LMA) (e.g. Witkowski & Lamont, 1991; Groom & Lamont, 1999; Osone *et al.*, 2008; Poorter *et al.*, 2012; Gil-Pelegrín *et al.*, 2017). Although the ecological and functional significance of LMA is still under debate (Kikuzawa *et al.*, 2013; Alonso-Forn *et al.*, 2020), it is widely accepted that an increase in LMA is a general response to environmental stress (Onoda *et al.*, 2011; Alonso-Forn *et al.*, 2020). Thus, many studies have reported a higher LMA under conditions of drought stress (Turner, 1994; Reich *et al.*, 1999; Jordan *et al.*, 2005), low winter temperatures (Ogaya & Peñuelas, 2007; González-Zurdo *et al.*, 2016; Niinemets, 2016), or under conditions of low

nutrient availability (Loveless, 1962; Niinemets *et al.*, 2009; Hassiotou *et al.*, 2010). A high LMA has also been traditionally interpreted as a trait that increases the structural resistance of leaves, as it may protect the leaf against herbivory or mechanical damage (Turner, 1994; Reich *et al.*, 1999; Sack *et al.*, 2013) and so expecting to achieve longer lifespans (Coley *et al.*, 1985; Reich *et al.*, 1999). However, Alonso-Forn *et al.*, (2020) recently revisited the role of these four factors (drought, cold, nutrient availability and leaf longevity) on sclerophylly within the genus *Quercus* L. and they concluded that no sole factor could explain all the variation observed in LMA, suggesting that these constraints may have a synergistic effect. Additionally, the mechanisms driving higher LMA at lower temperatures across species are still unclear (Alonso-Forn *et al.*, 2020), urging the consideration that both severity of cold and length of the cold season be considered together in ecological studies. This segregation between the duration (length of time during which stress occurs)

and intensity (extreme value or accumulated value of a climatic stress factor) has been previously incorporated in studies when analysing drought stress (e.g. Granier *et al.*, 1999), but rarely when considering cold stress.

LMA, defined above as the ratio between leaf mass and leaf area, is also related to leaf thickness (LT, µm) and leaf density (LD, mg cm^{-3}, i.e. dry mass per hydrated volume), given that LMA (g m^{-2}) = LT × LD (Witkowski & Lamont, 1991; Poorter *et al.*, 2009; John *et al.*, 2017). These structural variables can also depend on other parameters associated with leaf anatomy and chemical composition. While variation in LT is mainly attributed to changes in mesophyll thickness, LD is dependent (amongst other things) on the chemistry of cell walls and vein traits (Villar *et al.*, 2013; John *et al.*, 2017; Peguero-Pina *et al.*, 2017a). In addition, variation in LMA related to changes in LT or LD has been suggested to have two major and potentially contrasting consequences for leaf function, linked to leaf economic spectrum theory (Wright *et al.*, 2004; Shipley *et al.*, 2006). First, as performing photosynthesis is the main function of the mesophyll, we might expect that the increase in LMA would be associated with an increase in mesophyll thickness, to lead to a greater capacity for C assimilation per unit leaf area due to a higher amount of photosynthetic tissue per unit area (Niinemets, 1999; Niinemets, 2001; Peguero-Pina *et al.*, 2017a). Moreover, a higher instantaneous photosynthetic rate per unit leaf area would compensate for a shorter favourable season caused by a higher duration of stressful seasonally climatic events such as aridity or cold (Kikuzawa *et al.*, 2013; Peguero-Pina *et al.*, 2016a; Berdugo *et al.*, 2020). However, an increase in mesophyll thickness may increase the nitrogen content per unit area (Onoda *et al.*, 2017) and, therefore, increase the risk for herbivory (Meloni *et al.*, 2012). Thus, if changes in LMA are associated with changes in

photosynthetically competent tissues, thicker leaves that result in high LMA would achieve higher instantaneous assimilation rates per unit area compensating for a longer duration of climate stress events, but at the expense of a lower protection given by a stronger herbivore pressure (Scenario 1, Fig. 1). Second, changes in LMA associated with LD imply variation in the relative amount of cell types or variation in biomass allocation (Niinemets, 2001; Hassiotou *et al.*, 2010; Griffith *et al.*, 2016). For instance, a higher cell wall fraction with respect to cytoplasm should lead to an increase in LD (Poorter *et al.*, 2009) giving more structural resistance to the cell (Wyka & Oleksyn, 2014) needed under more intense climate stress events (Oertli *et al.*, 1990; Scholz *et al.*, 2012; Ding *et al.*, 2014; Alonso-Forn *et al.*, 2020), but inevitably reducing the efficiency of photosynthesis due to a reduction in cell wall permeability to CO_2 and N allocation in photosynthetic proteins (Onoda *et al.*, 2017). Similarly, higher vein density, which can also contribute to increasing LD (Alonso-Forn *et al.*, 2020), has been observed to be greater in more arid biomes (Sack & Scoffoni, 2013), to the potential detriment of reducing the amount of photosynthetic tissues. Therefore, higher LMA associated with an increase in LD, and thus of 'protective' traits against intense climatic stresses, could trigger an increase in leaf lifespan, compensating for the lower instantaneous C assimilation rate caused by an increase in the proportion of nonphotosynthetic tissues (Kikuzawa, 1995; Niinemets, 2001; Kikuzawa & Lechowicz, 2011; Scenario 2, Fig. 1).

In general, LMA and leaf functioning differ between deciduous and evergreen species. Deciduous and evergreen species indeed have been shown to differ in: (1) LMA values, with lower values in deciduous species (e.g. Castro-Díez *et al.*, 2000; Poorter *et al.*, 2009), (2) the leaf structural traits behind increases in LMA (de la Riva *et al.*, 2016), (3) biomass allocation (Puglielli
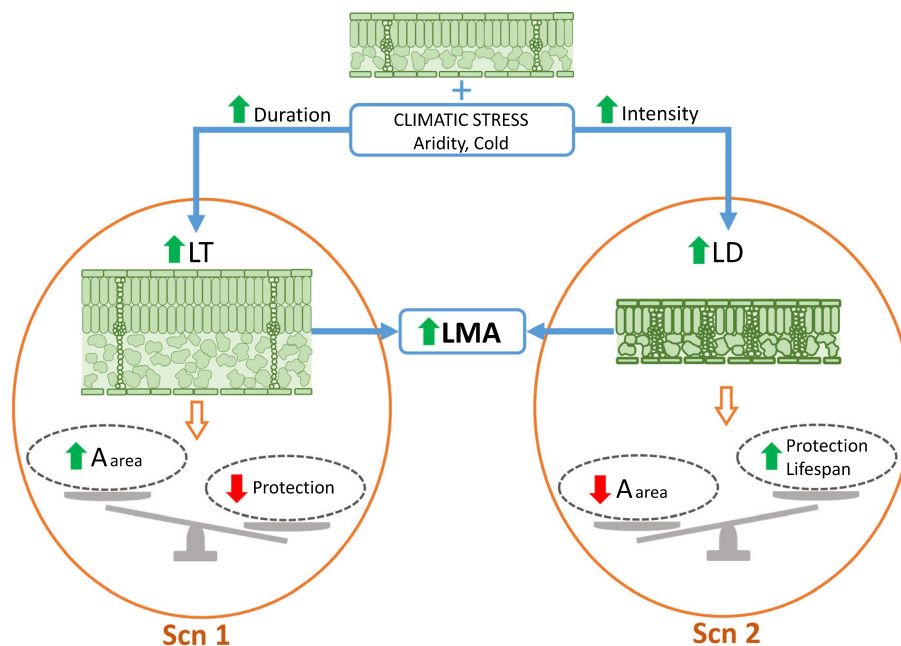

Fig. 1 Schematic representation of the two leaf mass per area (LMA) diversification scenarios. Scenario 1 (Scn 1): leaf thickness (LT) increases LMA to increase A$_{area}$ in order to compensate for a shorter favourable season. Scenario 2 (Scn 2): leaf density (LD) increases LMA as a protective mechanism against severe climatic factors. A$_{area}$, carbon assimilation per area.

et al., 2020), and (4) leaf longevity (Kikuzawa, 1991; Wright *et al.*, 2005; Kikuzawa *et al.*, 2013). For instance, a recent meta-analysis highlighted that, although variations in LMA were associated with LT and LD within both leaf habits, the variation across deciduous oak species was mainly due to variation in LT, while variation across evergreen oaks was better explained by variation in LD (Escudero *et al.*, 2017). In this sense, assuming that species from both leaf habits can fit into the two LMA diversification scenarios, the relative importance of Scenario 1 (LT increases LMA in order to compensate photosynthetically for a shorter favourable season) and Scenario 2 (LD increases LMA as a protective mechanism against severe climatic factors) within each leaf habit is also expected to be different. While variation of LMA within deciduous species could be more strongly associated with Scenario 1, variation within evergreen species might be more strongly associated with Scenario 2. Furthermore, it is imperative to investigate these scenarios within a phylogenetic framework, to help understand why plants have evolved certain leaf traits rather than others, which is a central question in evolutionary biology (Pigliucci, 2007).

In this study, we investigated the two LMA diversification scenarios in terms of several leaf traits enabling variation of LMA across deciduous and evergreen *Quercus* species. We quantified 14 leaf anatomical, morphological and chemical traits in a set of 40 deciduous and 45 evergreen *Quercus* species growing in a common garden in northern Spain. On the one hand, the genus *Quercus* is an excellent system to perform this study, as it not only minimises phylogenetic variation (compared with studies performed across diverse species), but also displays strong variation in LMA across species adapted to a large variety of climatic conditions, from those in nemoral–temperate areas to those in Mediterranean or tropical environments (Gil-Pelegrín *et al.*, 2017). Therefore, the genus *Quercus* constitutes a unique living laboratory to understand global adaptive patterns along the leaf economic spectrum in forest trees (Alonso-Forn *et al.*, 2020). On the other hand, by allowing the contribution of environmental variation to be ruled out as a factor in phenotypic variation, common gardens allow interspecific comparisons of traits that result from long-term adaptation to the environmental conditions where different species have evolved. LMA could therefore be analysed in relation to the species' native climate.

Four main objectives were addressed: (1) to quantify and contrast the diversification in leaf morphology, anatomy and chemical composition between deciduous and evergreen oaks; (2) to elucidate within each leaf habit which of the two components, LT or LD, was more relevant in the evolution of LMA in *Quercus*; (3) to identify for each leaf habit which anatomical and compositional traits are associated with increases in LT and LD; and (4) to investigate the role of climate in the differential diversification of LMA within deciduous and evergreen oaks. We hypothesised that diversification in LT was the main driver behind the diversification in LMA within deciduous oaks, whereas the diversification in LD would be more important within evergreen oaks. We also hypothesised that the duration of climatic stress would have a greater impact than stress intensity within deciduous species on LMA, but would have similar or less importance within evergreens.

Materials and Methods

Plant material

To perform this study, 85 oak species from across the Northern Hemisphere (Supporting Information Table S1) were sampled from living collections in the Jardín Botánico de Iturrarán (43°13′N, 02°01′W, 70 m asl, Gipuzkoa, Spain, www.iturraran.org). This common garden features temperate climatic conditions with a mean annual temperature of 14.5°C and a total annual precipitation of 1631 mm (Peguero-Pina *et al.*, 2016b). Current year, fully developed leaves were collected from southern-exposed branches of three trees per species during the early morning (07:00-09:00 h, solar time). All trees sampled were mature trees (15–25 yr old) to ensure leaf traits were measured at similar ontogenic changes (Puglielli *et al.*, 2020). Leaves were sealed in plastic bags and carried to the laboratory in portable coolers. As LT and LD variations can be explored through a series of anatomical and chemical traits (Villar *et al.*, 2013; John *et al.*, 2017), we used one set of 10 leaves per species (three or four leaves per tree) to measure leaf area, leaf mass and leaf fibre concentration, and another set of five leaves per species (one or two leaves per tree) to measure diverse anatomical parameters (details to be described later). Oak species were classified according to their leaf habit as evergreen (45 species), if they retained their leaves during the whole year and leaf lifespan was at least 12 months, and deciduous (40 species), if they lost all leaves during the winter season (Table S1). This classification was carried out with personal observations on the specimens of the common garden. We did not observe any species that retained their leaves during the whole year with a leaf lifespan lower than 12 months. Finally, climatic information based on the natural distribution range of each oak species was obtained to study the leaf traits in relation to climatic variables (to be described later).

Leaf mass per unit area and fibre concentration

Leaf area was measured by digitising 10 leaves per species and using IMAGEJ analysis software (http://rsb.info.nih.gov/nih-image/). Afterwards, leaves were oven dried for 3 d at 70°C and weighed using an analytical balance (Sartorius AG Model BP221S; Sartorius AG, Goettingen, Germany). LMA was then calculated as the ratio of the foliage dry mass to foliage area. Later, leaves were ground with a bead mill (TissueLyser II; Qiagen, Hilden, Germany) for fibre analysis. Hemicellulose, cellulose and lignin + cutin concentration were obtained by quantifying neutral detergent fibre and acid detergent lignin following the method of Goering & Van Soest (1970). Fibre concentration, obtained from a bulk of 10 leaves, was expressed as milligrams of fibre per gram of leaf dry mass. Abbreviations and units of these traits are shown in Table 1(a). The mean values (±SE) of leaf area and LMA, and the bulk values of fibre concentration are available in Table S1.

Table 1 List, units, abbreviations and association with the scenarios for (a) leaf traits and (b) climatic variables.

	Units	Abbreviation	Scenario
(a) Leaf traits			
Leaf mass per unit area	g m^{-2}	LMA	1, 2
Leaf thickness	µm	LT	1
Leaf tissue density	mg cm^{-3}	LD	2
Leaf area	cm^2	LA	1, 2
Hemicellulose concentration	mg g^{-1}	HC	2
Cellulose concentration	mg g^{-1}	CC	2
Lignin + cutin concentration	mg g^{-1}	LCC	2
Palisade mesophyll thickness	µm	PMT	1
Spongy mesophyll thickness	µm	SMT	1
Palisade mesophyll cell rows	count	PCR	1
Palisade cell length	µm	PCL	1, 2
Palisade cell width	µm	PCW	1, 2
Interveinal distance	µm	IVD	2
Bundle sheath extension width	µm	BSEW	2
(b) Climatic variables			
Mean monthly temperature	°C	t_m	1, 2
Monthly precipitation	mm	p_m	1, 2
Growing season. Number of months with $t_m > 5°C$	Month	GS	1
Period of full plant growth. Number of months with $t_m > 10°C$	Month	FPG	1
Arid period. Number of months with $t_m > 10°C$ and $2\,t_m > p_m$	Month	AP	1
Annual mean temperature	°C	T	1, 2
Minimum temperature of coldest month	°C	TMIN	2
Mean temperature of driest quarter	°C	TDRY	2
Annual precipitation	mm	P	1, 2
Arid intensity. Sum of $(2\,t_m - p_m)$ for months with $t_m > 10°C$ and $2\,t_m > p_m$		AI	2

Leaf cross-sectional anatomy

A transverse section ($c.\ 3 \times 2$ mm) was cut between the secondary veins of the middle part of each leaf. Sections were fixed in paraffin and embedded in paraffin blocks (Ruzin, 1999). Leaf cross-sections (20 µm in thickness) were obtained from the blocks using a rotary microtome (HM 350 S; Microm International GmbH, Walldorf, Germany). Cross-sections were deparaffinised and stained following the procedure of Johansen's safranin and fast green (Johansen, 1940; Ruzin, 1999). Afterwards, images of the cross-sections were taken using a digital camera (Optikam Pro 5; Optika Microscopes, Ponteranica, Italy) coupled to a light microscope (Optika B-600TiFL; Optika Microscopes, Ponteranica, Italy) (Fig. 2). Anatomical parameters including LT, palisade and spongy mesophyll thickness, number of palisade cell row layers, palisade cell size, interveinal distance (IVD) and bundle sheath extension width (BSEW), were then measured from the images using IMAGEJ software (Fig. 2). We measured one value per cross-section for each anatomical parameter, except for IVD and BSEW, for which we measured two values, and for palisade cell size for which we measured palisade cell length and width of

20 cells per cross-section. Finally, for each species, the LD was calculated as the ratio between LMA and LT (Witkowski & Lamont, 1991; Niinemets, 1999). Abbreviations of leaf anatomy traits, their units and their association with the scenarios are shown in Table 1(a). The mean values (±SE) of these anatomical traits for each species are available in Table S1.

Climatic variables

Climatic information (bioclimatic variables; mean monthly temperature, t_m; and monthly precipitation, p_m) of species natural distributions was obtained from the WorldClim database (http://www.worldclim.org/) using geographical distribution coordinates obtained from herbarium data (Gil-Pelegrín *et al.*, 2017). From this climatic information, we selected six climatic variables related to the duration and intensity of the seasonal stress, including cold and arid-related variables. Indeed, we used two climatic variables related to cold stress duration: the growing season and the period of full plant growth, defined as the number of months when monthly temperatures were higher than 5°C and higher than 10°C, respectively (Wypych *et al.*, 2017). We further selected one climatic variable related to arid stress duration: the aridity period, which we based off climatic diagrams (Breckle, 2002) that define aridity as the number of months where $2 \times$ monthly temperature (t_m) > monthly precipitation (p_m), considering only those months with mean temperatures higher than 10°C. We selected one climatic variable that characterises cold stress intensity, the minimum temperature of the coldest month. We finally selected two climatic variables characterising aridity stress intensity: the aridity intensity, defined as the sum of $(2t_m - p_m)$ for months with $2t_m > p_m$ and $t_m > 10°C$, and the temperature of the driest quarter (TDRY) (Table 1b). For a better understanding of TDRY, we identified the season of the year with the driest quarter by analysing the monthly precipitation of the localities for each species. This analysis showed that most of the species included in this study had their driest quarter either during summer (June, July, August) or during winter (December, January, February) (Fig. S1). Additionally, we include in the analyses the mean annual temperature and the mean annual precipitation as standard variables for comparative purposes with other studies. Abbreviations of climatic variables, their units and their association with each scenario are shown in Table 1(b). The mean values (±SE) of the eight climatic variables used in this study for each species are available in Table S2.

These climatic variables from all species were previously analysed using principal components analysis in order to identify the species within the eight climatic variables considered (Fig. S2). This previous analysis also showed that thermal-related variables (including the growing season and the period of full plant growth) weighed more in the first component, while arid variables weighed more in the second component.

Data analysis

First, Student's t-tests were used to compare the leaf trait values between deciduous and evergreen species in order to identify
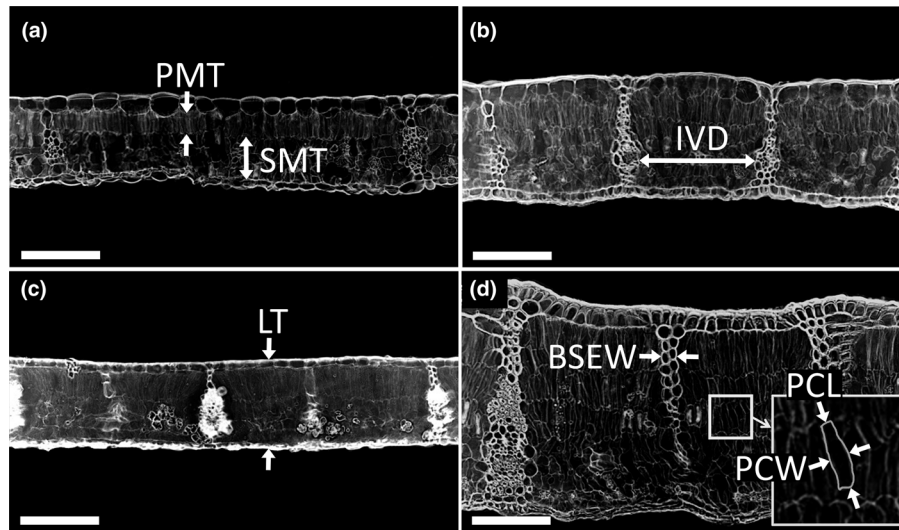
Fig. 2 Leaf mesophyll cross-sections of two deciduous (a, b) and two evergreen (c, d) *Quercus* species differing in leaf mass per area (LMA). (a) *Quercus robur* (LMA = 86 g m^{-2}). (b) *Quercus marilandica* (108 g m^{-2}). (c) *Quercus insignis* (90 g m^{-2}). (d) *Quercus miquihuanensis* (198 g m^{-2}). BSEW, bundle sheath extension width; IVD, interveinal distance; LT, leaf thickness; PCL, palisade cell length; PCW, palisade cell width; PMT, palisade mesophyll thickness; SMT, spongy mesophyll thickness. Bars, 100 μm.

their differences in terms of LMA, anatomy and chemical composition.

Second, evolutionary dynamics of traits following the approach of previous studies (Chatelet *et al.*, 2013; Scoffoni *et al.*, 2016) were examined with the aim of analysing within each leaf habit which of the two components, LT or LD, was more associated to the diversification of LMA in oaks. A phylogenetic tree was inferred from Hermida-Carrera *et al.* (2017), pruned to match species measured in this study. The phylogenetic tree was constructed using rbcL sequences from 85 *Quercus* species and *Fagus crenata* as a root species (see Table S3 for GenBank accession numbers), resulting in a tree similar to that shown by Hipp *et al.* (2020) and consistent in the assignment of species to subgenera (subgenus *Cerris* and subgenus *Quercus*) and sections (Denk *et al.*, 2017). Then, to visualise how increases in either LT and/or LD related to increases in LMA, we calculated the divergence of LT and LD from LMA across the study's oak species by first standardising the species means for all three traits to values between 0 and 100. Then, we calculated the absolute value of the difference between the standardised LMA and the standardised LT and LD values respectively for each species. As such, a divergence value close to 0 between LT and LMA across species would mean that both traits co-evolved very tightly. We also calculated the divergence of the two first climatic principal components (Fig. S2) from LMA, LT and LD in order to elucidate whether climate was tightly coordinated with these leaf traits across species.

Third, we compared the fit of three different models of trait evolution: a Brownian motion (BM) model, an Ornstein–Uhlenbeck (OU) model with a single global optimum (OU1) and an OU model with two optima either evergreen or deciduous species (OU2). The best fit model for each variable was chosen using the computed Akaike Information Criterion corrected for small sample size, and the corresponding variable was transformed appropriately. Then, relationships between leaf traits and climatic

variables across different species within each leaf habit were explored with regression and correlation analyses (Spss statistical package; SPSS Inc., Chicago, IL, USA). Analyses were performed using phylogenetically independent contrasts (PICS) (Harvey & Pagel, 1992). Interspecific differences in leaf traits were tested by one-way ANOVA (Spss).

All analyses were performed in R (v.3.6.1) using the packages Analysis of Phylogenetics and Evolution (Ape; Paradis *et al.*, 2004), Ornstein–Uhlenbeck models for phylogenetic Comparative Hypotheses (Ouch; Butler & King, 2004), and Analysis of Evolutionary Diversification (Geiger; Harmon *et al.*, 2008).

Results

Variation in anatomical and chemical composition across species and leaf habits

All leaf traits analysed exhibited strong interspecific variability, with leaf area showing the highest coefficient of variation (Table 2). LMA, palisade and spongy mesophyll thickness, and IVD showed intermediate coefficients of variation, whereas LD showed fewer differences between the different species. Hemicellulose and cellulose concentration and palisade cell size also showed relatively low variability (Table 2).

The coefficients of variation for LMA, LT, LD, leaf area, hemicellulose content, spongy mesophyll thickness and palisade cell length were 1.2–1.9-fold higher for evergreen than for deciduous species (Table 2). Evergreen and deciduous leaf habits also showed significant differences in the mean values of most leaf traits ($P < 0.05$; Table 2). Evergreen oaks had 1.4-fold higher LMA and LT than deciduous species, but 2.3-fold smaller leaf area. Cellulose concentration and lignin + cutin concentration were 1.3-fold and 1.1-fold higher,

Table 2 Mean value, maximum (max) and minimum (min) values, and coefficient of variation (CV) of leaf traits for deciduous (DEC) and evergreen (EVE) *Quercus* species.

	LMA	LT	LD	LA	HC	CC	LCC	PMT	SMT	PCR	PCW	PCL	IVD	BSEW
DEC (mean)	92±2 a	135±5 a	695±19 a	42±5 a	180±5 a	150±4 a	129±6 a	62±3 a	44±2 a	1.7±0.1 a	7.4±0.2 a	38±2 a	301±17 a	12.6±0.5 a
EVE (mean)	130±6 b	191±7 b	694±27 a	19±2 b	169±6 a	193±6 b	148±6 b	88±4 b	74±3 b	2.2±0.1 b	8.1±0.2 b	40±1 a	198±7 b	15.7±0.6 b
DEC (max)	129	232	1058	141	252	210	260	137	69	3	11	65	584	21.4
DEC (min)	53	87	487	3	119	104	74	24	28	1	6	23	173	5.4
EVE (max)	223	337	1205	75	270	290	264	161	135	3	11	56	346	25.1
EVE (min)	62	106	435	2	78	128	76	36	35	1	6	23	118	9.6
DEC (CV)	17	22	17	75	18	16	32	32	25	27	13	28	35	27
EVE (CV)	31	26	26	88	24	16	26	31	30	25	13	17	25	25
All species (CV)	32	30	22	91	22	20	29	36	39	29	14	22	39	28

Mean values are mean ± SE. $n_{DEC} = 40$. $n_{EVE} = 45$. Leaf traits notation as in Table 1. Different letters indicate statistically significant differences ($P < 0.05$) between deciduous and evergreen species. CV for all species is also shown.

respectively, in evergreen than in deciduous species. The larger thickness of evergreen species was due to the thicker mesophyll, mainly due to the 1.7-fold larger spongy mesophyll contribution (Table 2). There were also differences in the vascular bundle traits. Evergreen oaks showed a significant 1.5-fold lower IVD and, therefore, a higher vein density, and a significant 1.3-fold higher value of BSEW than deciduous oaks (Table 2). By contrast, LD, hemicellulose concentration and palisade cell length did not show significant differences between the two leaf habits ($P < 0.05$; Table 2).

Dynamics and models of trait evolution

Across species, LT and LD evolved in coordination with shifts in LMA, such that the direction and magnitude of evolutionary shifts in LT or LD corresponded to shifts in LMA (Fig. S3). However, LMA appears to have evolved in tighter coordination with LT than it did with LD; 83% of all deciduous oak species and 66% of the evergreen species belonging to subgenus *Quercus* showed a tighter coordination between LT and LMA (expressed as lower divergence between the traits; Fig. 3). By contrast, LD showed tighter coordination with LMA in *c*. 70% of the evergreen oaks within the subgenus *Cerris* (Fig. 3). Notably, high values of LMA could be found in any clade, but were preferably associated with evergreen species (Fig. 3).

Concerning the coordination between LMA and climate, 71% of the species showed a divergence value below 15, with at least one of the climatic principal components, that is LMA showed a very tight coordination with climate in most species analysed (Fig. 3). Across species from subgenus *Cerris*, LMA, LT and LD showed a slight tighter coordination with PC1 (the thermal component; Fig. S2) than they did with PC2 (the arid component; Figs 3,S2,S4). Within subgenus *Quercus*, LMA and LT also showed a slight tighter coordination with PC1, while LD showed a tighter coordination with PC2 (Figs 3,S4).

Comparing the fit of the three different models of trait evolution (BM, OU1 and OU2), the model that best fitted most of our trait variables (e.g. LMA, LT) was the OU2 model with two trait optimum (for deciduous and evergreen species) (Table S4). However, for some variables (e.g. LD) the fit of OU2 was similar or slightly lower than OU1 (single trait optimum).

Correlations in anatomical and chemical composition within leaf habits

Within deciduous species, LMA showed a positive association with LT (including its thickness components palisade and spongy mesophyll thickness and number of palisade cell rows), LD, palisade cell length, cellulose concentration and lignin + cutin concentration ($R = 0.436–0.643$, $P < 0.01$), and a negative association with IVD ($R = -0.596$, $P < 0.001$; Table 3a; Fig. 4). LT was positively associated with the thickness of the different tissue layers, with palisade cell rows, BSEW, cellulose and lignin + cutin concentration ($R = 0.316–0.737$, $P < 0.05$; Table 3a). LT was also negatively associated with LD and leaf area ($R = -0.408$ and -0.457, $P < 0.01$; Table 3a). LD showed
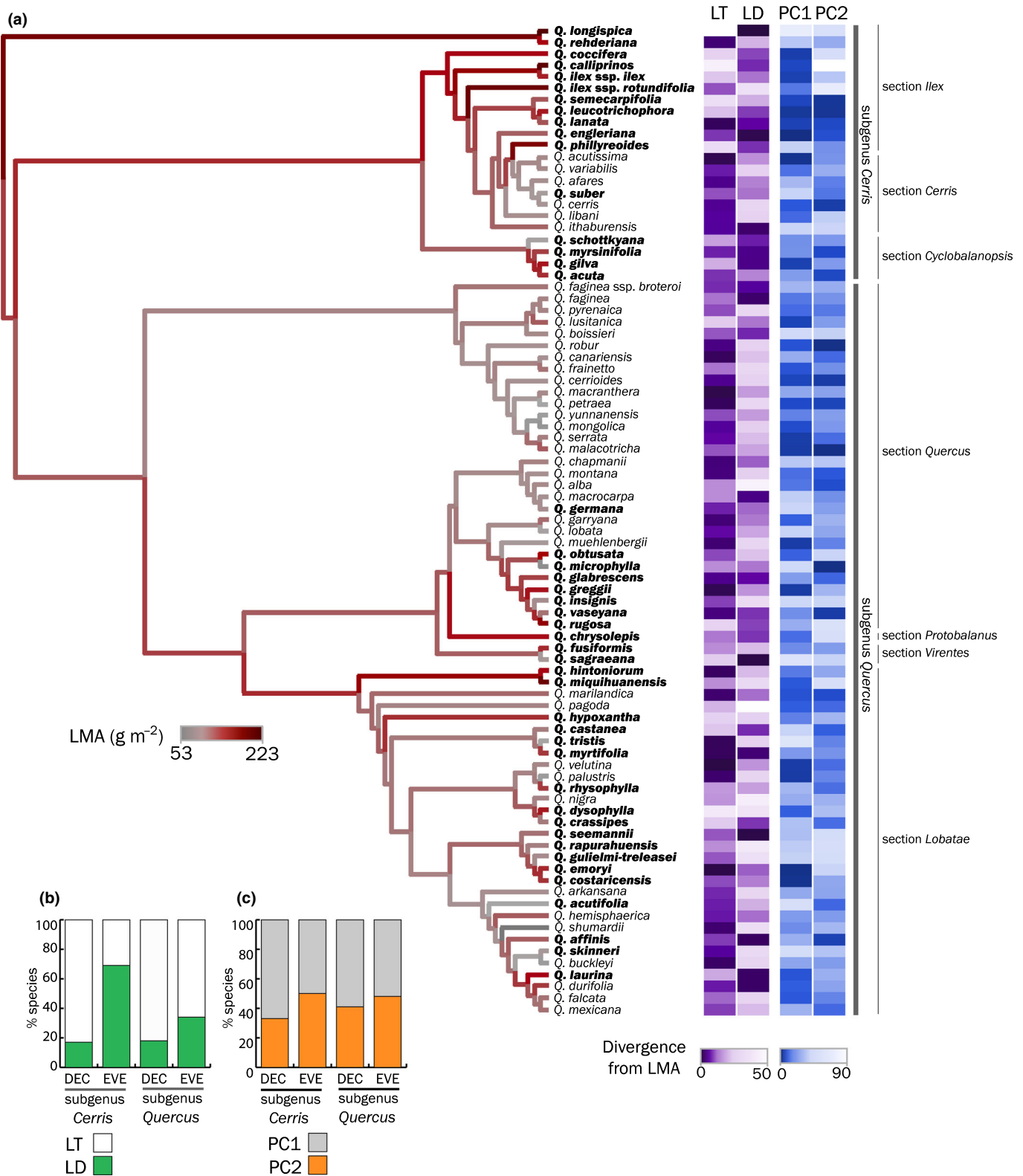
Fig. 3 (a) *Quercus* phylogenetic tree based on rbcL showing the evolution of leaf mass per area (LMA) in coordination with leaf thickness (LT), leaf density (LD) and climate. Darker colour for LMA indicates a higher value. Darker colours for LT, LD and the two first climatic principal components (PC1, PC2, see Supporting Information Fig. S2) indicate tight coordinated evolution with LMA (= low divergence) across our study's species. Bolded and nonbolded species names indicate evergreen and deciduous species, respectively. (b, c) Percentage of species showing a higher coordination (= lower divergence) between LMA and LT (white), LMA and LD (green), LMA and PC1 (grey), and LMA and PC2 (orange) for both leaf habits and both subgenera.

Table 3 Correlation coefficients between leaf traits for (a) deciduous (DEC) and (b) evergreen (EVE) *Quercus* species using phylogenetically independent contrasts (PICS).

(a) DEC	LMA	LT	LD	LA	(b) EVE	LMA	LT	LD	LA
LT	0.567***				LT	0.686***			
LD	0.449**	−0.408**			LD	0.405**	−0.354*		
LA		−0.457**			LA		−0.444**		
HC				0.572***	HC				
CC	0.525***	0.546***			CC	0.577***	0.474**		
LCC	0.465**	0.372*			LCC	0.317*			
PMT	0.575***	0.605***			PMT	0.817***	0.909***		−0.350*
SMT	0.590***	0.737***	−0.369*	−0.712***	SMT	0.528***	0.729***	−0.499*	−0.364*
PCR	0.436**	0.318*			PCR	0.774***	0.715***		
PCL	0.643***			−0.459**	PCL	0.536***	0.468**	−0.355*	−0.537***
PCW					PCW			−0.382*	
IVD	−0.596***		−0.489**	0.326*	IVD				
BSEW		0.316*	−0.475**	−0.513***	BSEW	0.584***	0.732***		−0.304*

Leaf traits notation as in Table 1. Significance levels: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

negative correlations with spongy mesophyll thickness, IVD and with BSEW ($R=-0.369$ to -0.489, $P<0.05$; Table 3a).

Within the evergreen species – as in the deciduous species – LMA showed positive associations with LT (including palisade and spongy mesophyll thickness and number of palisade cell rows), LD, palisade cell length, cellulose and lignin + cutin concentration ($R=0.317-0.817$, $P<0.05$), being also associated with BSEW ($R=0.584$, $P<0.001$; Table 3b; Fig. 4). LT was positively related to the thickness of the different mesophyll tissue layers, palisade cell rows and cell length, BSEW and to cellulose concentration ($R=0.468-0.909$, $P<0.01$) and negatively related to LD and leaf area ($R=-0.354$ and -0.444, $P<0.05$; Table 3b), similar to the deciduous species. Within the evergreen species, LD also showed a negative correlation with spongy mesophyll thickness and palisade cell size, including palisade cell length and width ($R=-0.366$ to -0.499, $P<0.05$; Table 3b).

Relationship of climate and leaf anatomy within leaf habits

Within deciduous species, LMA was positively correlated with the growing season ($R=0.376$, $P<0.05$) and to mean temperature of driest quarter (TDRY; $R=0.345$, $P<0.04$; Table 4a). Leaf area was also related to TDRY, but the trend was the opposite of that found for LMA (Table 4a). Additionally, leaf area also showed a negative correlation coefficient with minimum temperature of the coldest month (TMIN; $R=-0.393$, $P<0.05$; Table 4a). Surprisingly, LT within deciduous species was correlated with all climatic variables considered in this study, being positively associated with growing season, full plant growth period, aridity period, aridity intensity, mean annual temperature, TMIN and TDRY ($R=0.341-0.521$, $P<0.05$), and negatively associated with annual precipitation ($R=-0.483$, $P<0.01$; Table S4). LD was positively correlated with annual precipitation ($R=0.523$, $P<0.001$) and negatively correlated with aridity period and aridity intensity ($R=-0.465$ and -0.537, $P<0.01$; Table 4a).

For the evergreen species, LMA and LT were negatively correlated with the period of full plant growth ($R=-0.350$ and

-0.432, $P<0.05$), a trend that was opposite to that found in the deciduous species between these leaf traits and the growing season (Table 4a). LMA and LT were also negatively correlated with annual mean temperature, minimum temperature of coldest month and annual precipitation ($R=-0.471$ to -0.663, $P<0.01$), and positively correlated with aridity period ($R=0.381-0.581$; $P<0.05$; Table 4b). LMA was also positively correlated with aridity intensity ($R=0.401$, $P<0.01$; Table 4b). By contrast, leaf area showed positive correlations with minimum temperature of coldest month and annual precipitation ($R=0.645$ and 0.742, $P<0.001$) and negative correlations with aridity period and aridity intensity ($R=-0.420$ and -0.344, $P<0.05$), trends that were opposite to those found for LMA (Table 4b). Finally, LD was positively correlated with mean temperature of driest quarter, aridity period and aridity intensity ($R=0.316-0.494$, $P<0.05$; Table 4b).

Discussion

In this study, we analysed leaves from 85 *Quercus* species from across the Northern Hemisphere but living in a common garden. Our results showed that the evolution of leaf traits follows the OU2 model, which clearly segregates oaks in two main functional groups, deciduous and evergreen. Irrespective of the phylogeny or the species' natural habitats, these two groups differed in anatomical leaf attributes such as LMA, LT or leaf area (Table 2), as previously reported by other studies (Corcuera *et al.*, 2002; Escudero *et al.*, 2017). By contrast, they did not differ in LD despite their significant differences in leaf traits related to density, such as cellulose concentration, IVD and bundle sheet extension width (Table 2). This fact could be due to the thicker spongy mesophyll of evergreens that may promote a larger amount of intercellular airspaces, counteracting the effect of these other leaf traits on LD (Escudero *et al.*, 2017).

Taking into account the segregation given by the OU2 model, we evaluated variation in LMA related to changes in LT or LD within each leaf habit according to two scenarios (Fig. 1) linked to the leaf economic spectrum theory (Wright *et al.*, 2004).
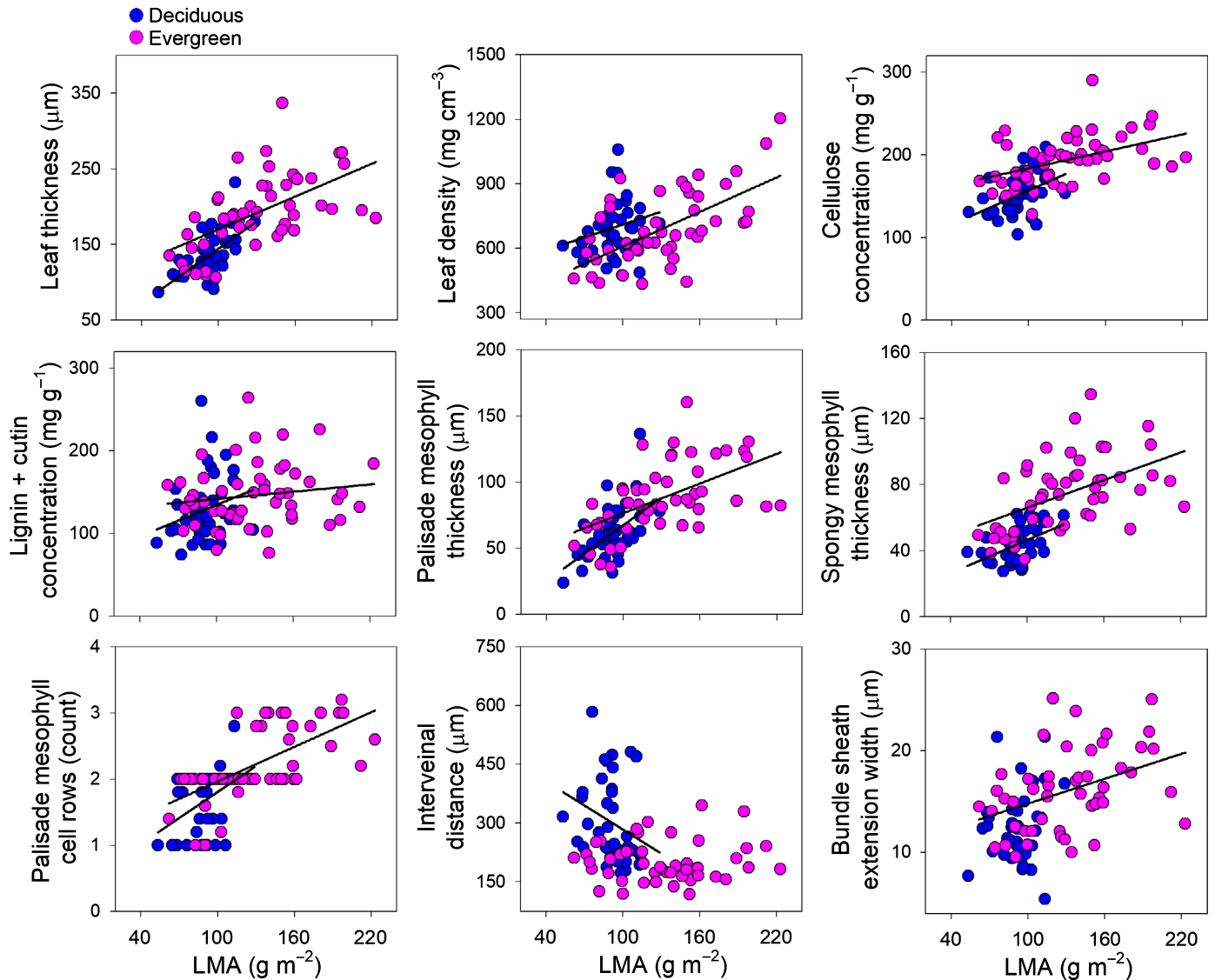
Fig. 4 Relationships between leaf mass per area (LMA) and key leaf traits for deciduous (blue) and evergreen (pink) *Quercus* species. Regression coefficients for the phylogenetic independent contrasts (PICS) are shown in Table 3. Each circle is the mean value of one particular species.

Scenario 1: LMA associated with photosynthetically competent tissues to compensate a shorter favourable season

As leaves are essentially organs utilised for photosynthesis (Turner, 1994), Scenario 1 contemplated that an increase in LMA would be achieved by thicker leaves in order to increase the instantaneous carbon assimilation rates per unit area to compensate for shorter favourable seasons in stressful climatic environments (Fig. 1). When this scenario was analysed within evergreen oak species, our results effectively showed that evergreen oaks living in environments with lower periods of full plant growth (i.e. longer cold periods) and/or with higher aridity period, increased LMA by increasing LT (Table 4; Fig. 5). This suggested that evergreen oaks inhabiting higher altitudes (e.g. the subtropicals *Q. rehderiana* and *Q. rugosa*), with longer dry periods (e.g. the subtropical *Q. obtusata*) or at higher latitudes under more

continental climatic conditions and longer dry summers (e.g. the Mediterranean oaks *Q. chrysolepis* and *Q. ilex* ssp. *rotundifolia*), tended to exhibit a higher LMA due to a higher LT than those evergreen oaks inhabiting lower altitudes, lower latitudes and with a minimal hot dry season (e.g. the subtropicals *Q. sagraeana, Q. seemannii* or *Q. insignis*; Fig. S2). By contrast, when analysing Scenario 1 within deciduous species, our results showed that the deciduous oaks with higher LMA due to a higher LT inhabited environments with shorter cold periods (which was opposite to our prediction in Scenario 1) but with longer and more intense aridity periods. This opposite pattern related to the length of the growing season found for deciduous species compared with the evergreens seemed to be consistent with global patterns (Wright *et al.*, 2005) and prediction based on a cost–benefit model (Kikuzawa *et al.*, 2013). However, for deciduous species, a longer aridity period may counteract the effect of a shorter cold period by shortening the favourable season (Peguero-Pina *et al.*, 2016a).

Table 4 Correlation coefficients between leaf traits and climatic variables for (a) deciduous (DEC) and (b) evergreen (EVE) *Quercus* species using phylogenetically independent contrasts (PICS).

(a) DEC	LMA	LT	LD	LA	(b) EVE	LMA	LT	LD	LA
GS	0.376*	0.427**			GS				
FPG		0.341*			FPG	−0.350*	−0.432**		
AP		0.521***	−0.465**		AP	0.581***	0.381*	0.327*	−0.420**
T		0.370*			T	−0.598***	−0.638***		
TMIN		0.325*		−0.393*	TMIN	−0.471**	−0.611***		0.645***
TDRY	0.345*	0.502**		−0.348*	TDRY			0.494***	
P		−0.483**	0.523***		P	−0.495***	−0.663***		0.742***
AI		0.445**	−0.537***		AI	0.401**		0.316*	−0.344*

Leaf traits and climatic variables notation as in Table 1. Significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

This counteracted effect, together with the positive association between aridity intensity and LT, pointed to aridity stress as the main driver increasing LT within deciduous species. In this sense, a higher LT not only would increase the instantaneous carbon assimilation rates per unit area when water is more readily available, but would also improve water use efficiency under drought conditions (Wright *et al.*, 2001; Gil-Pelegrín *et al.*, 2017). Examples of such deciduous species inhabiting arid environments could be the subtropical *Q. mexicana* or the European and North American Mediterranean-type oaks *Q. ithaburensis* and *Q. garryana* (Fig. S2).

The increase in LT that promoted an increase in LMA was given by increasing the mesophyll thickness mainly through increasing the number of cell layers, both within deciduous and evergreen (Table 3; Fig 4), which has been previously observed across diverse species (de la Riva *et al.*, 2016; John *et al.*, 2017). This increase in mesophyll thickness has the potential to increase photosynthetic rates per unit leaf area (A_{area}) (Niinemets *et al.*, 1999). However, this assumption should be made carefully as there are other leaf traits that could modify A_{area} (Peguero-Pina *et al.*, 2017b). For example, increases in cell wall thickness that promote higher cellulose concentration (Terashima *et al.*, 2006) may result in a reduction in A_{area} (Peguero-Pina *et al.*, 2017a,c). Therefore, the positive correlation found in this study between palisade mesophyll thickness and cellulose concentration, both in deciduous ($R = 0.333$, $P < 0.05$) and evergreen ($R = 0.607$, $P < 0.001$; data not shown) species, could counteract the presumed positive effect of a higher mesophyll thickness on A_{area}. Additionally, the presumed increase in A_{area} could also be negated by an increase in nonphotosynthetic tissues. Our results showed that a higher LT is coordinated with a higher BSEW in both leaf habits, and reduces the amount of photosynthetic tissues (Table 3; Fig. 5). This relationship suggests that thicker leaves might have higher mesophyll structural reinforcement, which may prevent thickness shrinkage during dehydration processes (Sancho-Knapik *et al.*, 2011). In these two cases (a higher cell wall thickness and thicker bundle sheath extensions), LT and density could be considered coupled rather than being a trade-off relationship (Onoda *et al.*, 2017). Finally, the assumed higher A_{area} of oak species with higher LT may also be diminished at the leaf level by the reduction of leaf area (Tables 3, 4). Given that reduction in leaf size is a key modification that allows plants to

withstand water deficit (Baldocchi & Xu, 2007), oaks inhabiting arid environments could increase LT to increase A_{area} in order to compensate for their smaller leaf area (Peguero-Pina *et al.*, 2016a).

Scenario 2: LMA as a protective mechanism against severe climatic factors

Protection will require further investment that would promote tougher, harder and stiffer leaves that might increase the leaf lifespan of a particular species (Turner, 1994; Read & Sanson, 2003). Therefore, our Scenario 2 considered that an increase in LMA would be associated with denser leaves, in order to increase their resistance under stressed climatic conditions, and therefore to increase their lifespan (Fig. 1). When analysing Scenario 2 within evergreen oaks, our results showed that those species living in environments with higher aridity intensity and higher temperature of the driest quarter (TDRY), increased LMA by increasing LD (Table 4b; Fig. 5). As TDRY can mostly occur during winter or during summer (Fig. S1), it seems that those evergreens with denser leaves are those inhabiting environments with drier summers (e.g. the Mediterranean *Q. calliprinos* and *Q. coccifera*, Fig. S2). By contrast with evergreens and contrary to our Scenario 2, an increase in aridity intensity within deciduous oaks was directly associated with an increase in LMA through increases in LT, as mention above, but with decreases in LD (Table 4a). This result suggested that, within deciduous oaks, higher aridity promotes thicker and less dense leaves (e.g. in the Mediterranean-type *Q. garryana* or *Q. lusitanica*), which may improve A_{area} by increasing CO_2 mesophyll conductance (Niinemets *et al.*, 2009).

The positive relationship found between LMA and LD within each leaf habit, as reported before by other authors (Groom & Lamont, 1997; Poorter *et al.*, 2009; Read *et al.*, 2016), was negatively influenced by the increase in spongy mesophyll thickness, both in evergreen and deciduous oaks. In other words, a higher spongy thickness might promote a larger volume of intercellular air spaces that could decrease the overall LD and, therefore, weaken other relationships. However, we found that a higher LD was associated with a reduction in cell size within evergreen species, and with a lower IVD (i.e. higher vein density) through a decrease in leaf area within deciduous species, anatomical
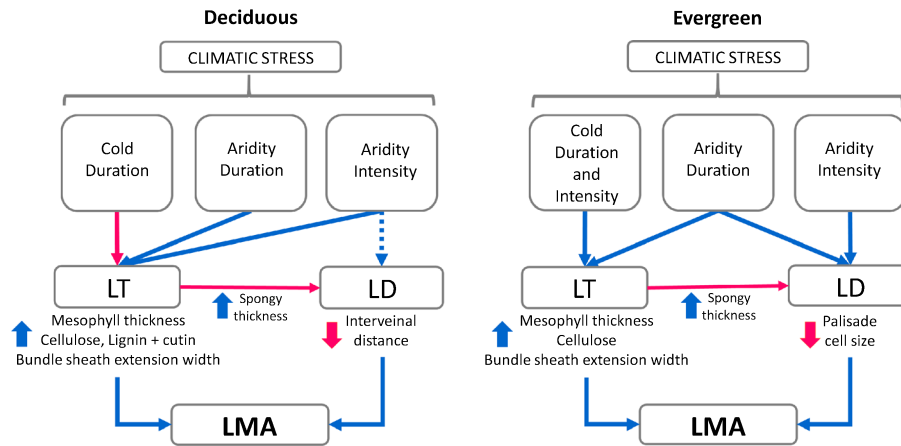
Fig. 5 Schematic representation of the main associations found for anatomical (Table 3) and climatic variables (Table 4) for deciduous and evergreen *Quercus* species. A blue arrow indicates a positive association, while a pink arrow indicates a negative association between two traits. The dashed line indicates an indirect association. LD, leaf density; LMA, leaf mass per area; LT, leaf thickness.

modifications that have been reported as protective adaptations to withstand water stress (Oertli *et al.*, 1990; Scoffoni *et al.*, 2011; Ding *et al.*, 2014; Nardini *et al.*, 2014). Furthermore, we can also highlight other protection mechanisms within evergreen oaks found in this study related to LT. As abundant intercellular air spaces provide room to accommodate extracellular ice (Wyka & Oleksyn, 2014), the higher LT due to a higher spongy mesophyll thickness found in evergreen species inhabiting colder environments (Tables 3, 4; Fig. 5), may protect evergreen species against frost damage. Additionally, as low winter temperatures may cause damage by absorption of excess light under limiting conditions for photosynthesis, being thicker may also reduce the probability for photodamage (Peguero-Pina *et al.*, 2009). To our knowledge, there has been no previous report of cold intensity as a determinant factor of interspecific variation in LMA, although Wright *et al.* (2005) observed similar effects of mean annual temperature in evergreen species. However, other authors observed intraspecific trends in the LMA of evergreen species also related to gradients in winter temperatures (Ogaya & Peñuelas, 2007; Mediavilla *et al.*, 2012; González-Zurdo *et al.*, 2016; Niinemets, 2016). Moreover, minimum temperature of the coldest month in our study was also positively related to the length of the growing season ($R = 0.8$, $P < 0.001$; Fig. S5), suggesting that environments with colder winters are also sites with shorter growing seasons, and therefore sites with longer winters (van Ommen Kloeke *et al.*, 2012). Taking into account that our results showed that an increase in LMA may constitute an adaptation against both cold duration and cold intensity in evergreen oaks (Fig. 5), further research is needed to partition these two effects and pinpoint whether a higher LMA is a direct response to one and/or the other.

Diversity in evolutionary trends

Our results showed that across most of the oaks included in this study, LMA evolved in greater coordination with LT than with LD (Fig. 3). This trend was found in deciduous oaks and in the evergreen species from the subgenus *Quercus*, mainly belonging to

the *Lobatae* and *Quercus* sections (Denk *et al*, 2017). By contrast, in the evergreen oaks from the subgenus *Cerris* – mainly belonging to sections *Ilex* and *Cyclobalanopsis* – LMA converged more with LD (Fig. 3). This discrepancy within evergreen oaks, could be due to the ancestral origin of each group. While the ancestors of sections *Lobatae* and *Quercus* are inferred to have occurred at higher latitudes (Hipp *et al.*, 2018; Cavender-Bares, 2019), the ancestors of *Ilex* and *Cyclobalanopsis* may have inhabited lower latitudes (Barrón *et al.*, 2017; Jiang *et al.*, 2019). Consequently, ancestors of *Lobatae* and *Quercus* could have increased LMA by increasing LT in order to achieve higher carbon assimilation rates to compensate for a shorter favourable season typical of higher latitude environments where daylength varies considerably from summer to winter solstice. By contrast, ancestors of *Ilex* and *Cyclobalanopsis* could have increased LMA by increasing LD in order to protect their leaves against stress factors (e.g. mechanical damage) and, therefore, to increase their leaf lifespan under environments with longer growing seasons, typical of lower latitudes. The possibility that different oaks ancestors could depend primarily on the length of the growing season could help to explain why the thermal climatic component– which also includes the growing-season length variables – was slightly more important in explaining variation in LMA than the arid component. Further research is needed to fully test these new hypotheses. However, our results clearly showed that LMA was tightly coordinated with climate, independently of leaf habit or the climatic component considered. This reinforces climate as an important driver of leaf functioning in oaks; this was recently pointed out by Ramírez-Valiente *et al.* (2020) and supported by work in oaks across altitudinal gradients (Fallon & Cavender-Bares, 2018).

Finally, our data also revealed that within *Lobatae* oaks, LMA changed according to the latitude of the species distribution (Fig. S6). All *Lobatae* oaks inhabiting higher latitudes (*c.* 40°) are deciduous, and tend to have lower LMA values than *Lobate* oaks found at middle latitudes (*c.* 25°). This variation within deciduous *Lobatae* oaks is tightly coordinated with changes in the length of the growing season and temperature. At middle latitudes (*c.* 25°), there is a shift in leaf habit in our set of species, from

deciduous to evergreen, all having higher LMA than those living at lower latitudes (*c.* 10–15°). According to our data, the variation in LMA within evergreen *Lobatae* oaks is associated with changes in temperature and precipitation of species climatic niches (Fig. S6). The association of LMA with latitude within *Lobatae* oaks could be a consequence of the radiation from a high-latitude ancestor (Hipp *et al.*, 2018) towards lower latitudes across North and Central America (Cavender-Bares, 2019). However, these outcomes within *Lobatae* oaks should be considered carefully, as not all *Lobatae* oaks were included in our study. Further research is needed to confirm this result and to elucidate the main drivers of leaf trait evolution within the others infrageneric groups.

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Author contributions

DS-K, TGA-A, JJP-P and EG-P planned and designed the research. DS-K, DA-F and JJP-P performed the measurements. DS-K, AE, SM, JZ and AM generated phylogenetic data. DS-K, AE, SM, CS, JZ, JC-B, DA-F, JPF, JJP-P and EG-P analysed climatic and phylogenetic data. DS-K, AE and CS drafted the manuscript. All authors edited the manuscript with valuable inputs.

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Data availability

Data that support the findings of this study are openly available in TRY at http://doi.org/10.17871/TRY.64.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Season location of the driest quarter of the year.

Fig. S2 Principal components analysis of the climatic variables.

Fig. S3 Oak phylogenetic trees showing the evolution of leaf mass per area, leaf thickness and leaf density.

Fig. S4 Divergence of the climatic principal components from leaf thickness and density.

Fig. S5 Relationship between minimum temperature and length of the favourable season.

Fig. S6 Relationships within section *Lobatae* between leaf mas per area, latitude and climatic variables.

Table S1 Leaf habit and leaf traits mean values of the study's species.

Table S2 Climatic variables mean values of the species' climatic origins.

Table S3 *Quercus* GenBank accession numbers for rbcL gen.

Table S4 Model fitting using Brownian motion and the Ornstein–Uhlenbeck models.

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