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## Research Paper

# Genetic differentiation in functional traits among European sessile oak populations

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**The vulnerability of forest species and tree populations to climate change is related to the exposure of the ecosystem to extreme climatic conditions and to the adaptive capacity of the population to cope with those conditions. Adaptive capacity is a relatively under-researched topic within the forest science community, and there is an urgent need to understand to what extent particular combinations of traits have been shaped by natural selection under climatic gradients, potentially resulting in adaptive multi-trait associations. Thus, our aim was to quantify genetic variation in several leaf and woody traits that may contribute to multi-trait associations in which intra-specific variation could represent a source for species adaptation to climate change. A multi-trait approach was performed using nine *Quercus petraea* provenances originating from different locations that cover most of the species' distribution range over Europe and that were grown in a common garden. Multiple adaptive differences were observed between oak provenances but also some evolutionary stasis. In addition, our results revealed higher genetic differentiation in traits related to phenology and growth than in those related to xylem anatomy, physiology and hydraulics, for which no genetic differentiation was observed. The multiple associations between those traits and climate variables resulting from multivariate and path analyses suggest a multi-trait association largely involving phenological and growth traits for *Q. petraea*.**

**Keywords:** adaptive capacity, embolism resistance, climate change, phenology, plant ecophysiology, plant functional traits.

## Introduction

Climate change and the associated increase in mean temperature and reduction in precipitation are expected to induce significant shifts in species' distributions due to drought-induced population diebacks (Bertin 2008, Allen et al. 2010, Delzon et al. 2013, Allen 2014). This has raised important concerns not only about our ability to predict population mortality and its impact on ecosystem function (Cailleret et al. 2017), but also about the capacity of species to adapt in a timely manner to the expected warmer and drier climates (Corlett and Westcott 2013, Sáenz-Romero et al. 2017, González-Muñoz et al.

2018). These concerns are especially relevant to forest species given, on one hand, the rapid rate of environmental change and, on the other, the long life span of most tree species (Aitken et al. 2008). Therefore, crucial questions for evaluating and predicting the consequences of ongoing climate change are (i) which key traits can evolve within a few generations allowing the adaptation of trees to climate change, and (ii) how fast can such adaptation occur in response to a changing environment?

During the last decade, many studies have focused on addressing these questions by monitoring woody plants under controlled or induced environmental changes (Hoffmann and

Sgrò 2011, Franks et al. 2014). As a result, different traits have been identified that respond phenotypically to temperature, thus making them potential targets for microevolution (e.g., Tatsuya et al. 2010). Phenology is one of the most well-known sensitive indicators of climate change. In oaks, previous studies have shown genetic differentiation in bud phenology that has resulted in a shift of 2–3 days in spring and 0.3–1.6 days in autumn per decade over the last 50 years, extending the growing season (Vitasse et al. 2009). Although the benefits can vary across species, it has been shown that individuals that flower early produce flowers throughout the entire growing season, thus maximizing fitness compared with late-flowering individuals (Anderson et al. 2012). However, much less is known about the genetic determinism of leaf functional traits in oaks, such as stomatal density, leaf size and leaf thickness, that have significant influence not only on net carbon gain but also on plant water balance. The genetic determinism of xylem and hydraulic traits that are related to the hydraulic failure of the plant water transport system due to embolism formation is also largely unknown in oak despite embolism formation being considered to be one of the main mechanisms leading drought-induced plant mortality (Brodribb and Cochard 2009, Salmon et al. 2015, Urli et al. 2015, Choat et al. 2018). Understanding intra-specific variation in these traits would therefore help us evaluate the capacity of tree species to face and adapt to new environmental conditions induced by ongoing climate change.

In this study, we investigated intra-specific variation of numerous traits related to leaf phenology and physiology with the aim to better understand the potential for adaptation of oak populations. Indeed, genetic variation could help ensure survival of at least some individuals or populations during extreme events, buffering the population or the species against extinction (Meireles et al. 2017).

Some of these traits have previously been assessed in common garden experiments and exhibited clinal genetic variation along geographic gradients as a result of diversifying selection (Vitasse et al. 2009, Alberto et al. 2011). Thus, divergent intra-specific profiles for 'intrinsic' water-use efficiency (Farquhar and Richards 1984) have been linked to the distribution of genotypes across gradients in air humidity and soil water availability for different species (Pennington et al. 1999, Cregg and Zhang 2001, Aletà i Soler et al. 2009). In addition, a previous study in oaks carried out in a common garden with populations from different locations along an elevation gradient showed how genetic differentiation accounted for up to 28% of total variation in traits, such as leaf mass area and nitrogen content for European oak and beech (Bresson et al. 2011). However, some studies reported no evidence of genetic differentiation for growth traits between populations along an aridity gradient (Deacon and Cavender-Bares 2015, Ramírez-Valiente et al. 2017). Environmental changes can also affect wood and water

metabolism-related traits, such as wood density, vulnerability to embolism and water-use efficiency (Mencuccini 2003, Schume et al. 2004, Brienen et al. 2011). In fact, genetic differences in both wood density and vulnerability to embolism have been reported for some species (Arnold et al. 2004, Sotelo Montes and Weber 2009, David-Schwartz et al. 2016) but not for others (Lamy et al. 2011).

In this contribution, we purposely implemented a multi-trait approach by considering different functional traits related to either growth, phenology, structure (wood density) or physiology. Our aim was to investigate whether diversifying selection along climate gradients triggered specific associations of multiple traits whose genetic variation could represent a source for adaptation. Although previous studies have shown that some traits may follow clinal genetic variation along climatic gradients, their pattern of genetic variation has never been investigated collectively in a single and large common garden. We studied nine *Quercus petraea* L. provenances diverging from a common source population (from the last glacial period, 15,000 years before present) and originating from different locations that cover most of the species' distribution range over Europe and grown in a common garden. We focused on a paneuropean oak species, *Q. petraea* (sessile oak). This species has been extensively monitored at the molecular level to document spatial and temporal differences across Europe but to a much lesser extent at the phenotypic level, which was our main aim. In addition, *Q. petraea* is an ideal species for drawing adaptive inferences based on genetic divergence. Indeed, recent microevolutionary patterns of variation were shown to be less blurred by historical or demographic noise in oaks in comparison with other species, due to their very low level of subdivision on the basis of neutral markers (Firmat et al. 2017).

## Materials and methods

### Common garden experiment

This study utilized a common garden experiment that was planted in 1989 and 1993 in the Forêt Domaniale de Sil-légny (France), which contains 107 sessile oak provenances (Ducousso et al. 1996). From these, nine provenances diverging from a common source population and representing different climatic regions within the distribution range of the species in Europe, from Northern Germany to Southern France, were selected for this study (Figure 1 and Table 1, climate data source: Worldclim; period: 1960–90). The initial density of the plantation was 1904 individuals per hectare (spacing 3 × 1.75 m) with each provenance replicated from 10 to 15 plots with 24 trees per plot. At the time of the study, most of the trees were 25 years old and 10 m tall on average (see details about plantation years in Table 1). Although sample size differed between the different traits studied because of varying

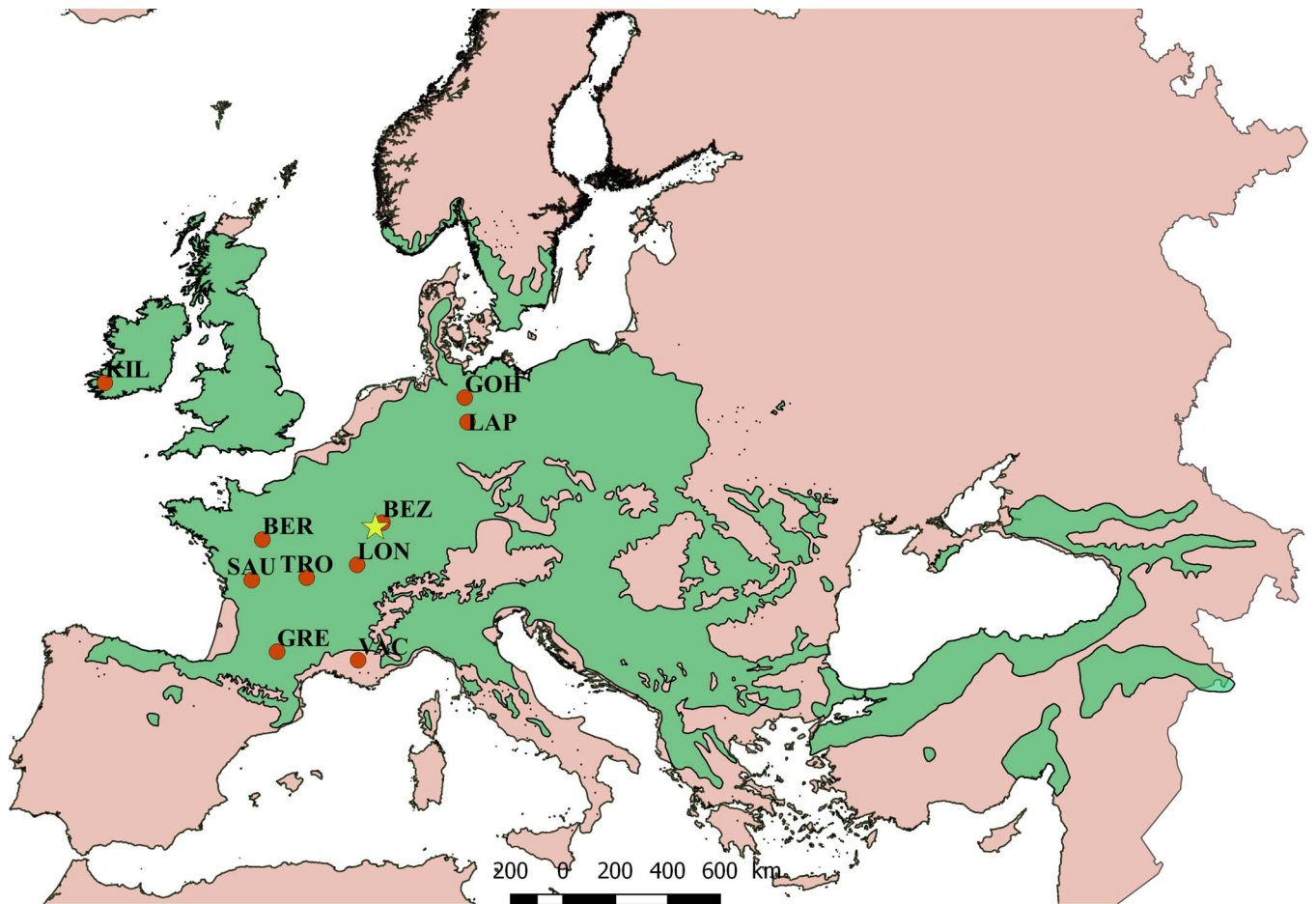


Figure 1. Distribution of *Q. petraea* in Europe. Red dots indicate the origin of the provenances grown in the common garden located in Sillegny (yellow star) and used in this study (see Table 1 for climate information and abbreviation of each site).

complexity of measurements, all traits were evaluated in the exact same set of trees per provenance.

#### Phenology and tree height

We monitored spring and fall phenology in the nine selected provenances in 2014. Leaf unfolding (LU) in spring was monitored every 10 days in 25 individuals per provenance distributed in 7–12 replicated plots. Leaf senescence (LS) was monitored twice in late September and mid-October 2014 in the same individuals monitored for LU. Phenological observations were made using binoculars (magnifying power: 109) at a distance of approximately 10 m from each tree, by the same observer. In spring, we recorded the development stages from bud dormancy to LU, using a scale with five intermediate stages according to Vitasse et al. (2009). We considered that a bud had reached LU stage when at least one of its leaves was fully unfolded. At the tree level, LU date was determined when 50% of the buds had reached this threshold. In fall, due to the low number of field campaigns, we were not able to estimate a date of LS and therefore used the score of LS obtained during the October

campaign (the September campaign was not discriminative enough). The senescence score corresponds in percentage to the amount of non-functional leaves, either colored or fallen, according to Vitasse et al. (2009). Height of all individuals was measured in January 2015.

#### Mean leaf area, specific leaf area and wood density

Mean leaf area (MLA,  $\text{mm}^2$ ) and specific leaf area (SLA,  $\text{m}^2 \text{kg}^{-1}$ ) were determined for 22–28 trees per provenance randomly selected in 7–12 blocks per provenance and with at least one tree per block. Between 10–15 fully expanded and non-damaged leaves were collected per tree. To avoid any possible effects of different light exposure within the crown on leaf traits, all samples were collected from the upper and outer part of the crown to ensure a similar light exposure among them. All leaves were collected within 48 h, starting on 14 June 2014. They were collected from a single branch per tree using a pole pruner or by shooting them down. Immediately after being collected, leaves were placed in sealed plastic bags to avoid desiccation, stored in cooling boxes, and transported to the lab. Once in the lab, six

Table 1. Climatic data, location, altitude and aridity index of the studied *Q. petraea* provenances.

Provenance	Country	Latitude (DD)	Longitude (DD)	Altitude (m)	Mean temperature (°C)	Precipitation (mm)	Aridity index	Common garden plantation year
St Sauvant (SAU)	France	46.38	0.12	155	11.35	838	0.91	1989
Killarney (KIL)	Ireland	52.01	-9.50	50	10.14	1374	2.09	1990
Grésigne (GRE)	France	44.04	1.75	310	11.97	806	0.81	1990
Bézange (BEZ)	France	48.76	6.49	275	9.54	761	0.99	1993
Bercé (BER)	France	47.81	0.39	165	10.71	709	0.85	1993
Longchamp (LON)	France	47.26	5.31	235	10.85	793	0.96	1993
Tronçais (TRO)	France	46.68	2.83	245	10.55	752	0.86	1993
Vachères (VAC)	France	43.98	5.63	650	10.77	779	0.92	1993
Göhrde (GOH)	Germany	53.10	10.85	85	8.49	629	0.90	1993
Lappwald (LAP)	Germany	52.26	10.99	180	8.64	598	0.83	1993

to eight leaves per tree were scanned to measure the area of each individual leaf using a desktop scanner (Expression 10000 XL, Epson, Nagano, Japan) and WinFolia software (Regent Instruments Inc., Quebec, Canada). For determining the SLA, the dry mass of leaves was measured after drying them in an oven at 65 °C until a constant mass was reached. The SLA was assessed as the ratio of the leaf area to its mass. Individual leaf data were later averaged over all leaves per tree.

Wood density was estimated using X-ray imagery (Polge 1966) on a section of dry branch. One ca 10-cm-long branch segment per individual was collected from 4 to 16 individuals per provenance. As for leaves, to avoid any possible effects of different light exposure within the crown on wood density, all branches were collected from the upper and outer part of the crown to ensure similar light exposure conditions among them. All branches had the same age (2 years old) within and between provenances. For each sample, we used a double-bladed saw to cut a transverse section with a constant thickness of 2 mm. Wood density was measured on the transverse section by using an X-ray image calibration procedure. Sections were exposed to X-rays and were then scanned with a microdensitometer. Images were analysed using Windendro (Guay et al. 1992) to obtain two radial density profiles per section. Ring limits were determined automatically, checked manually, and then corrected with this software. We then calculated mean wood density ( $D$ ,  $\text{g cm}^{-3}$ ).

### Stable C and N isotopes analysis

After MLA and SLA measurements, the same leaves were used for determining the carbon and nitrogen content (C and N,  $\text{g kg}^{-1}$ , respectively) and isotopic discrimination ( $\Delta^{13}\text{C}$  and  $\Delta^{15}\text{N}$  for C and N, respectively). The dry samples were ground to powder using a wood grinding sample system (Labman, Stokesley, North Yorkshire, UK) and put into a tin capsule for mass spectrometry. The C and N isotope ratios as well as C and N contents were measured on 3-mg samples at the Microbiology & Agronomics Platform at INRA Reims (France) by using an

isotope ratio mass spectrometer (Delta Advantage, Thermo Scientific, Bremen, Germany). The carbon isotopic composition expressed as  $\delta^{13}\text{C}$  in ‰ (Craig 1957) was then converted into carbon discrimination  $\Delta^{13}\text{C}$  in ‰ (Farquhar and Richards 1984). The  $\Delta^{13}\text{C}$  values were corrected for the Suess Effect (decrease in  $\delta^{13}\text{C}$  of atmospheric  $\text{CO}_2$  since the beginning of industrialization) resulting from the emission of fossil carbon dioxide, which is depleted in  $^{13}\text{C}$  (Francey et al. 1999, McCarroll and Loader 2004) even if the sampling has been done at the same date.  $\Delta^{13}\text{C}$  can be related to the ratio of  $\text{CO}_2$  assimilation ( $A$ ) to stomatal conductance ( $g_s$ ), also named the intrinsic water-use efficiency.

### Leaf vein and stomatal density

Vein density (VD, total vein length per  $\text{mm}^2$  of leaf area) was determined from paradermal sections of five fresh leaves (one leaf per tree, five trees per provenance) similar to those used for MLA and SLA. Sections were prepared and measured following the protocols described by Carins Murphy et al. (2012). In brief, this involved removing the adaxial epidermis and palisade tissue, clearing all pigments with bleach, and measuring VD from slide mounts of the sections using image analysis of digital photomicrographs (five fields of view per section). Stomatal density (total stomata per  $\text{mm}^2$  of leaf area) was also determined from cuticles (1 per leaf and five fields of view per cuticle) prepared and measured following the protocols of Carins Murphy et al. (2012).

### Vulnerability to embolism

Due to the complexity of these measurements, vulnerability to xylem embolism was determined in three out of the nine selected provenances and one extra provenance (originally from Ireland) for which the aridity of the provenance origin was much lower (Table 1). For each provenance, 15 individuals randomly selected in seven to nine blocks per provenance and with at least one tree per block were evaluated in June/July 2015. These four provenances were selected according to their aridity index (AI),

which was calculated as:

$$AI = MAP/MAE$$

where MAP and MAE represent the mean annual precipitation and mean annual potential evapotranspiration, respectively. The four provenances selected were Grésigne (Southeastern France) Killarney (Southern Ireland), Vachères (Southwestern France), and Göhrde (Northern Germany), and represent different climatic regions, ranging from a dry Mediterranean region in France to a continental temperate climate in Germany (plus the additional provenance from a cool and humid oceanic climate in Ireland).

Xylem vulnerability to embolism was measured using the Cavitron technique (Cochard 2002, 2005) at the Caviplace laboratory (GENOBOIS platform, INRA-University of Bordeaux, France). To prevent artefactual losses in hydraulic conductance due to the induction of embolism during the sample preparation (Torres-Ruiz et al. 2015) or the presence of open vessels in the samples (Torres-Ruiz et al. 2017a), >2-m-long branches were collected from the trees (one branch per tree), wrapped in moist paper and plastic bags to kill transpiration and transported to the laboratory. Once in the lab, branches were progressively recut under water to release the xylem tension according to Torres-Ruiz et al. (2015) and to adjust them to a 1-m-long length. Branches were debarked at both ends and installed in a large cavitron equipped with a 1-m-diameter custom-built honeycomb rotor (DGMeca, Gradignan, France) (Lobo et al. 2018). Several branches were used to test the presence of open vessels by air injection at two bars, and none of them presented open vessels in 1-m-long branches. Samples were spun for 3 min at a given speed to decrease the xylem pressure progressively at its center from  $-0.8$  to  $-10.5$  MPa (those pressures correspond to centrifugation rotation from 764 to 2768 r.p.m., respectively). Vulnerability curves to embolism were generated by plotting the percentage loss of hydraulic conductivity (PLC) at the different target pressures applied and fitting a sigmoidal equation (Pammenter and Van der Willigen 1998). Mean  $P_{50}$  values, i.e., the xylem pressure inducing 50% of PLC, were obtained by averaging the values of 13–15 samples per provenance. The  $P_{50}$  value is commonly used as a proxy for tree drought resistance: the lower the  $P_{50}$  value, the more drought tolerant the species (Delzon 2015, Torres-Ruiz et al. 2017b).

### Statistical analyses

Differences in phenological, physiological, anatomical and hydraulic traits among the nine provenances of *Q. petraea* were tested with a generalized linear mixed model (MIXED procedure, restricted maximum likelihood (REML) method in SAS, version 9.4, SAS Institute, Cary, NC, USA) where plots and provenances were respectively treated as fixed and random

factors. The provenance effect was further assessed using a log likelihood ratio test from the full and reduced models (Littell et al. 2007). The data were checked to satisfy the assumptions of normality and homogeneous variance prior to analyses. The ratio of the provenance variance component to total variance was estimated according to Vitasse et al. (2009) using the VARCOMP procedure with the restricted maximum likelihood (REML). These analyses used the following statistical model:  $Y_{ijk} = \mu + P_i + b_j + (Pb)_{ij} + \varepsilon_{ijk}$ , with  $Y_{ijk}$  being the observed trait of the seedling  $k$  from the provenance  $i$  and block  $j$ ,  $\mu$  the overall mean of the analysed characters,  $P_i$  the random effect of provenance  $i$ ,  $b_j$  the fixed effect of block (here replicated plot)  $j$ ,  $(Pb)_{ij}$  the interaction between provenance  $i$  and block  $j$ , and  $\varepsilon_{ijk}$  the residual variation including the effect of tree  $k$  belonging to combination  $ijk$ . Variances of random effects (provenance  $\sigma_P^2$ , interaction  $\sigma_{bP}^2$ , residual  $\sigma_\varepsilon^2$ ) were also computed. The overall differentiation among provenances ( $D$ ) was calculated as the ratio (%) of the variance component of provenance to total variance estimated by analysis of variance, i.e., as  $\sigma_P^2 / (\sigma_P^2 + \sigma_{bP}^2 + \sigma_\varepsilon^2)$ , and varied from 0 to 100.  $D$  is an analog of  $Q_{st}$  (Spitze 1993), which is the genetic differentiation of quantitative traits ( $Q_{st} = \sigma_P^2 / (\sigma_P^2 + 2\sigma_A^2)$ ), where  $\sigma_A^2$  is the within provenance additive variance. In our study based on provenance and not descendant test,  $\sigma_A^2$  could not be estimated and we thus used the overall within-population phenotypic variance (i.e.,  $D$ ) rather than the overall genetic variance as it is for  $Q_{st}$  (Vitasse et al. 2009, Bresson et al. 2011).

The variability of each trait was evaluated by estimating both the intra- and inter-provenance coefficient of variation. The  $CV_{intra}$  was calculated for each provenance and then averaged at the species level, while the  $CV_{inter}$  was obtained from the between-provenance standard deviation and the overall mean value.

Weighted linear regression analyses were used to assess (i) relationships between the functional traits that showed significant differences among provenances and (ii) whether genetic variation was explained by the local environmental conditions (i.e., latitude, temperature and precipitation) of the provenances' origin. In addition, relationships between phenological and functional traits with climate variables were investigated using principal component analysis (PCA) in order to identify patterns of inter-relationships. Finally, a path analysis was carried out to test different conceptual models that could explain how the climate at the original location of each provenance determines the variance of the different functional traits. Those models were based on different hypotheses considering only the traits that were the most influenced by temperature and precipitation according to the PCA analyses (Figure S3 available as Supplementary Data at *Tree Physiology* Online). The best model based on the Akaike information criterion (AIC) would therefore provide valuable information about the associations of climate and multi-trait values.

Table 2. Genetic differentiation ( $D$ ) among provenances, coefficient of variation (%), intra- and inter-provenances, and log likelihood ratios (LLR) for the different phenological and functional traits. The LLR are given as provenance was treated in the GLMM as a random factor.  $P_{50}$  = xylem pressure inducing 50% of loss in stem conductivity;  $D$  (%) = the ratio of the variance component of provenance to total variance estimated by analysis of variance ( $\sigma_P^2 / (\sigma_P^2 + \sigma_{bP}^2 + \sigma_e^2)$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Trait	df	LLR	$D$	CV <sub>intra</sub>	CV <sub>inter</sub>
<i>Phenology</i>					
LU	8	45.30***	62	4	6
LS	8	18.90***	32	57	64
<i>Growth</i>					
Height	8	7.20**	0	9	10
<i>Physiology</i>					
SLA	8	5.80*	79	15	16
MLA	8	0.60	5	22	23
$\Delta^{13}\text{C}$	8	0.00	0	4	4
$\Delta^{15}\text{N}$	8	0.00	0	11	13
C content	8	0.00	0	5	5
N content	8	0.00	0	10	11
Stomatal density	8	0.00	0	16	16
<i>Hydraulics</i>					
$P_{50}$	3	0.00	0	9	10
<i>Anatomy</i>					
Vein density	8	0.00	0	9	8
Wood density	7	0.70	0	14	14

## Results

### Genetic differentiation

Significant genetic differentiation between provenances was observed in 4 out of the 13 studied traits (Table 2). The provenances differ in both the timing of leaf spring and autumn phenology (LU and LS, respectively). Tree height (H) and SLA were also significantly different between provenances, whereas a lack of genetic differentiation was found for MLA,  $\Delta^{13}\text{C}$ ,  $\Delta^{15}\text{N}$ , leaf C and N content, stomatal density,  $P_{50}$ , vein density and wood density. Overall, genetic differentiation for the different traits evaluated ranged from 0 up to 79. Thus, SLA and LU amounted the highest values with 79 and 62, respectively, whereas it was weaker for LS (32) and MLA (5). A null (0.0) overall differentiation was observed for all the other traits (Table 2). Most traits showed, to a greater or a lesser extent, higher coefficient of variation across provenances than within provenances (Table 2). Only leaf vein density showed a slightly higher variation within (9) than across provenances (8).

### Genetic clines

Correlations between traits and the climate and latitude of provenance origin reveal some relevant trends (Figure 2). Thus, positive and negative trends were observed between LU and both latitude and temperature of the provenance origin, respectively. Thus, the earliest provenance to begin leaf flushing

was the one originating from the southern margin (latitude 43.98°), whereas the latest was one from northern Germany (latitude 53.18°). Both the mean annual maximum and minimum temperatures correlate with the date of LU, with earlier budburst in provenances from warmer areas. In addition, late LU was observed in areas with lower precipitation. Thus, for the Grésigne provenance with a mean annual precipitation of 806 mm LU occurred in day of year (DOY) 93.7, whereas for Göhrde with a precipitation of 629 mm year<sup>-1</sup>, it occurred in DOY 104.2, i.e., 10.5 days later than for Grésigne. Concerning leaf shedding, while significant genetic differentiation was observed between provenances (Table 2), no significant cline with the climate of provenances was detected (Table S1 available as Supplementary Data at *Tree Physiology Online*). A similar pattern as in LU was observed for SLA, with higher values in provenances originating from higher latitudes and colder and drier areas. Thus, mean SLA ranged from 9.95 to 12.39 m<sup>2</sup> kg<sup>-1</sup> within a range in temperature of 17.14–4.42 °C and in total annual precipitation of 838–598 mm. Tree height, however, was not significantly affected by latitude or maximum temperature, but significantly increased with increasing minimum temperature and precipitation. Thus, trees are taller (up to 10.74 m) in provenances with higher minimum temperatures (6.8 °C) and annual precipitation (838 mm) (Figure 2). Interestingly, xylem resistance to embolism showed similar  $P_{50}$  values across the four provenances evaluated (i.e., no genetic differentiation, Figure 3A), but a significant correlation with mean annual temperature (MAT), showing increased resistance to embolism with higher MAT (Figure 3B). Surprisingly, AI did not correlate with any of the evaluated traits (Table S1 available as Supplementary Data at *Tree Physiology Online*).

### Relationships between phenological and functional traits and climate variables.

Only a few significant correlations were observed between those traits exhibiting a provenance effect (Table 3). Thus, tight correlations were observed between LS and H (Figure 4A) and between SLA and LU (Figure 4B). Thus, lower SLA was observed in those provenances with an earlier flushing, with Grésigne having the lowest SLA value (9.9 m<sup>2</sup> kg<sup>-1</sup>) and flushing on DOY 94. On the contrary, the highest SLA values was observed for Lappwald (12.4 m<sup>2</sup> kg<sup>-1</sup>), which flushed on DOY 103. The correlation between LS and H was observed (Figure 4A) reporting a lower percentage of colored leaves on in taller trees. The PCA showed how the first two principal components together explained 59.2% of the variance (Figure 5A). Thus, Axis 1 (variance, 37.5%) was strongly associated with the main climate variables, i.e., maximum and minimum temperature and precipitation, as well as with functional leaf traits LU,  $P_{50}$ , SLA and N content (Figure 5B). Axis 2 (variance, 21.7%), on the contrary, was more defined by the variance in LS, MLA,  $\Delta^{15}\text{N}$ , and tree H than by climate variables (Figure S2 available as

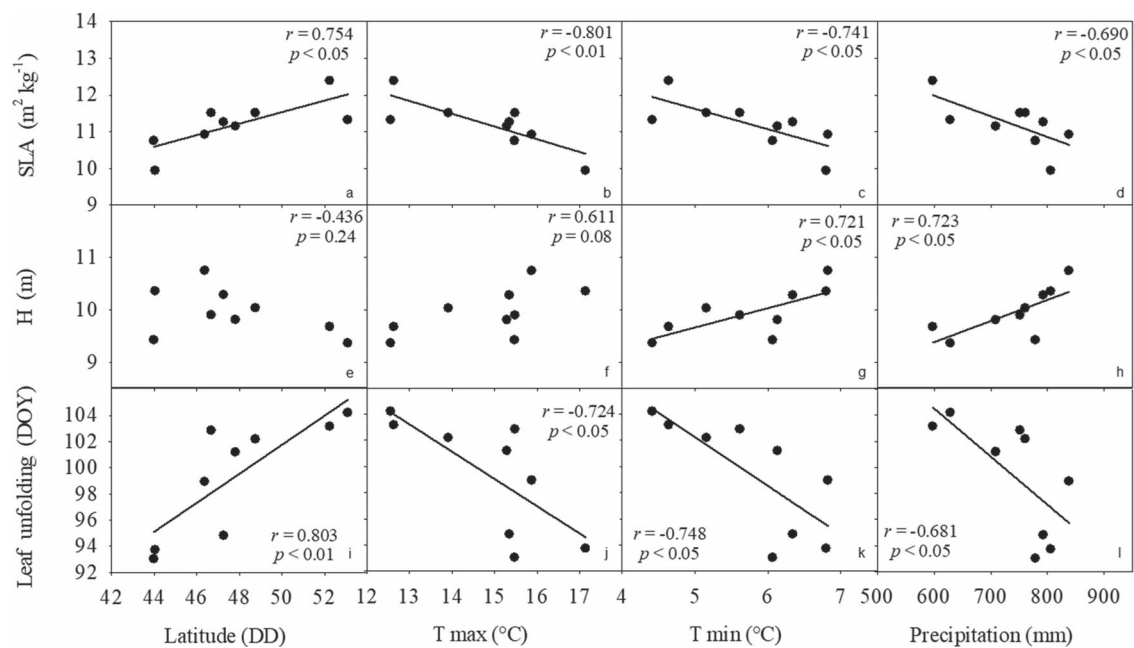


Figure 2. Specific leaf area (SLA), mean tree H, and LU of the nine provenances studied in the common garden at Sillegny versus latitude, mean annual maximum and minimum temperature (Tmax and Tmin, respectively), and precipitation.  $r$  indicates Pearson product-moment correlation;  $p$  indicates significance levels of the  $P$ -values of the slope.

Table 3. Statistics for the Pearson correlation at the individual level to each pair of traits exhibiting a provenance effect. Values in each cell indicate the Pearson correlation coefficient. Asterisk (\*) indicates a significant correlation between two traits ( $P < 0.01$ ).

	Tree height	LU	LS
SLA	-0.079	0.226(*)	0.079
Tree height		-0.094	-0.448(*)
LU			0.001

Supplementary Data at *Tree Physiology* Online). When running the path analyses to detect associations between the main climate variables and the functional traits, we found that the minimum temperature at the provenance origin seemed to be the most relevant trait determining their LU, SLA and N content values (Figure 6).

## Discussion

Nine provenances of *Q. petraea* originating from an extensive portion of the species' overall distribution in Europe showed significant genetic differentiation in traits related to leaf phenology, morphology and growth, but not in traits related to xylem anatomy and hydraulics when grown in a common garden. Phenology and some functional traits, such as  $P_{50}$ , also showed significant clines with the latitude and climate of provenance origin. Results from PCA and path analyses reported multiple associations between climate variables and both phenology and

functional traits that would be driven by a diversifying selection along the studied climatic gradient.

### Multi-trait associations

The effect of climate change on phenological traits has been extensively studied (Peñuelas et al. 2002, Gordo and Sanz 2005), especially the effect of temperature on the timing of LU and flowering, which both occur earlier as temperature rises (Doi and Katano 2008, Chung et al. 2013). However, unlike this study, many previous studies have been carried out 'in situ' and thus do not allow for any attribution of trait variation to either plasticity or genetic differentiation. Our results from trees grown in a common garden showed a significant cline between LU and the climate of provenance origin, with earlier bud burst in provenances from the warm margin. Therefore, local adaptation of phenology to the environmental conditions is observed for *Q. petraea*. Similar results have been already reported for different tree species including oaks (Menzel and Fabian 1999, Matsumoto et al. 2003, Vitasse et al. 2009), showing the important role of local temperature in driving adaptation of the growing season length. This may partially explain the significant differences in growth (i.e., tree height) observed between provenances, although a more complete understanding of the role of the vegetative growth period length and assimilation rates on the final growth of the populations would require a more focused study. In fact, our results also agree with a previous study by Kuster et al. (2014) in which three different oak species were grown on two different soils and exposed to air warming and drought.

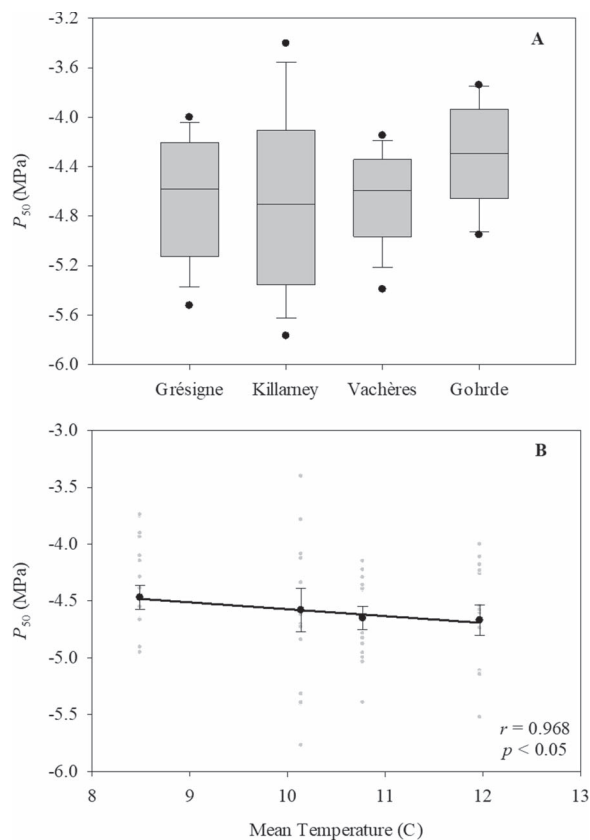


Figure 3. (A) Box plot of  $P_{50}$  (i.e., pressure at which 50% loss of stem conductivity occurs) for each of the four provenances in which resistance to embolism was evaluated. Between 13 and 15 different branches from different trees were used for each provenance. No significant differences between provenances were observed in  $P_{50}$  ( $p = 0.21$ ). (B) Significant correlation between  $P_{50}$  and mean temperature. A linear regression was fitted to the whole data set ( $r$ , Pearson product-moment correlation;  $p$ , significance levels of the  $P$ -values of the slope). The error bars represent  $\pm$  the standard error.

Their results showed earlier LU for trees exposed to higher air temperatures, leading to an earlier start of shoot growth, in that they estimated an advance in LU of 1–3 days  $^{\circ}\text{C}^{-1}$ . Our results also agree with those reported by Vitasse et al. (2009), who observed negative genetic clines in LU with increasing temperature at the origin of the provenance for *Q. petraea*. More recent studies (Alberto et al. 2011, Firmat et al. 2017) reported a similar genetic cline for both germination and bud burst timing with provenance elevation in *Q. petraea*, showing that spring leaf phenological traits are critical for local adaptation in oaks. Those genetic variations in leaf phenology observed in oaks are probably due to differences in heat requirement for bud burst rather than differences in chilling associated with leaf shedding (Dantec et al. 2015).

In contrast to other studies (Vitasse et al. 2009), a clinal trend with temperature was not observed for LS in our study despite the significant variation in this trait among provenances and despite showing the highest between-provenance variation

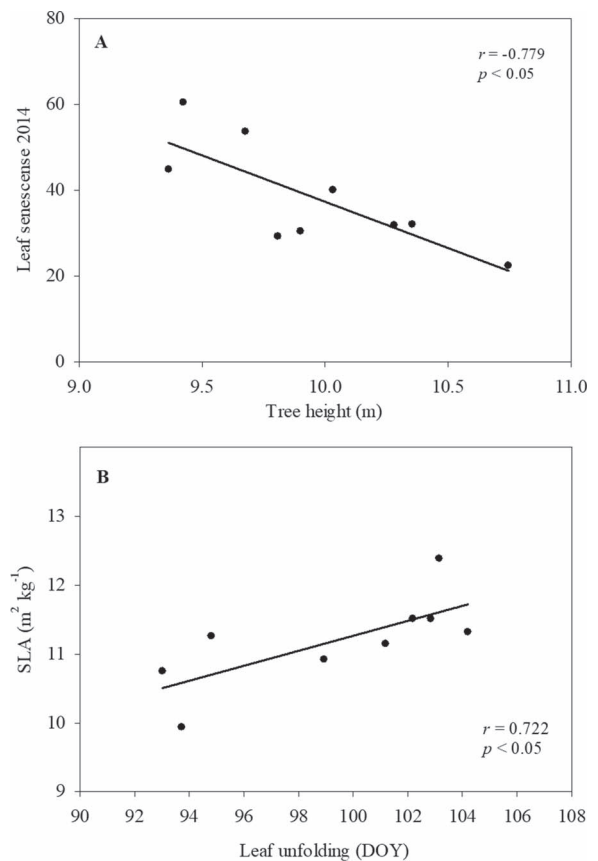


Figure 4. Significant correlations between pairs of phenological and ecophysiological traits. Each point represents the mean value of 22–28 trees per provenance. Linear regressions were fitted for the whole data set ( $r$ , Pearson product-moment correlation;  $p$ , significance levels of the  $P$ -values of the slope).

value. This is confirmed by the results from the PCA showing that, contrary to LU, the variation in LS is barely determined by the temperature and precipitation regimes of the provenances' origin.

Significant genetic differentiation in SLA, a trait directly related to light conditions and nutrient availability (Milla et al. 2008), was observed between provenances, indicating genetic variation among provenances induced by the climate at their original location. Similar to 'in situ' observations from Bresson et al. (2011) for *Q. petraea*, we observed a negative correlation between SLA and both temperature and precipitation. In fact, results from the PCA show exactly this: a high influence of the minimum and maximum temperatures and precipitation not only on SLA but also on other traits, such as  $P_{50}$  and LU. Intraspecific variation in SLA with air temperature and precipitation has been already reported for other species including maize and wheat for which, under N fertilization and irrigation controlled conditions, responses to both climate variables explained 43.7 % of the variation in SLA (Martin et al. 2018). Earlier LU would increase leaf lifespan, which has been reported to be correlated with SLA. Thus, across species, Reich et al. (1991)



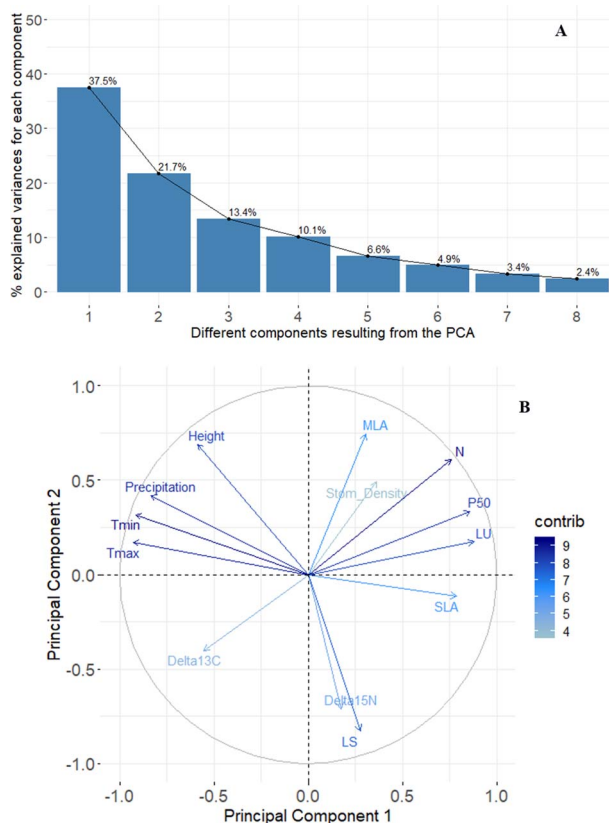


Figure 5. (A) Percentage of variances explained by the different axes (components) resulting from the PCA. (B) Variables factor map on the first two axes of the PCA performed on the different climate variables and functional traits. For facilitating its evaluation, only those traits with a higher contribution to component 1 and 2 ( $\cos^2 > 0.25$ ) are shown. The variables factor map including all traits is included in the supplementary information of the article (Figure S1 available as Supplementary Data at *Tree Physiology Online*).

showed that species with short leaf lifespan generally have thinner leaves (high SLA) than those with longer leaf lifespan, which agrees with our results within species. As the authors suggested, the correlation between leaf lifespan, and therefore LU and SLA, would result from different allocation trade-offs between species to enhance productivity, nutrient conservation or defense. This could also be the case at the intra-specific level and may explain observed differences between provenances. The relevance of the adaptation of SLA to climate resides in the fact that it may contribute to a species' ability to adjust to different air temperatures and precipitation regimes (Albert et al. 2010, Long et al. 2011), because SLA is a key plant functional trait reflecting the trade-off between resource capture and conservation (Wright et al. 2004). Interestingly, no genetic differences were found in leaf size between provenances. This finding links with a recent study evaluating the bivariate leaf size–climate relationships for 7670 plant species from 682 sites worldwide that shows how day and night time leaf-to-air temperature differences are key to explaining the latitudinal

gradient in leaf size (Wright et al. 2017). Thus, considering the results from Wright et al. (2017) and our results, latitudinal changes in leaf size would likely be due to phenotypic plasticity rather than to genetic variation.

### Lack of evidence for local adaptation

Leaf vein and stomatal density are directly linked with plant transpiration (Brodribb et al. 2007, Franks and Beerling 2009) and also highly influenced by environmental factors (Woodward and Bazzaz 1988, Uhl and Mosbrugger 1999), with higher stomatal and venation densities in drier areas (Herbig and Kull 1992, Carlson et al. 2016). Furthermore, both traits tend to remain proportional during leaf acclimation to light intensity and VPD in woody angiosperm species (Brodribb and Jordan 2011, Carins Murphy et al. 2012, 2016). However, the determinism of these traits has received limited attention so far (Zhu et al. 2012). Assuming these traits varied between provenances in situ, which is likely given the capacity for acclimation in vein density to variation in evaporative demand among the upper and lower canopy in the closely related *Quercus rubra* (Zwieniecki et al. 2004), our results suggest that leaf capacity to acclimate to different conditions via changes to these anatomical traits is not translated into genetic differences. In fact, there is no evidence for genetically driven effects on stomatal density in *Q. petraea* L. and *Fagus sylvatica*, although the phenotypic effect of temperature is large (Bresson et al. 2011). However, contrary to this, vein density was found to be genotypically fixed in *Quercus variabilis* (Zhu et al. 2012). A possible explanation for our observation that these traits that tend to show large phenotypic variation appear to have not responded to divergent selection here is that the populations sampled could have not spanned all the variation within the distribution area of this species. However, an efficient phenotypic plasticity response could also have had an important role in the lack of variation observed for these traits since it would lead to convergence among populations in trait expression when grown in a common garden. An alternative explanation is that other traits not measured in this study but that also contribute to leaf water supply and demand varied among provenances. For example, stomatal size, along with stomatal density, determines stomatal conductance to water vapor. Likewise, the distance between vein tips and stomata, along with the horizontal spacing of veins, determines the length of the post-vein pathway from the end of the vascular system to the sites of evaporation in the leaf, which itself is correlated with leaf hydraulic conductance (Brodribb et al. 2007). In this study differences were found in SLA but not in leaf size, implying that leaf thickness varied among provenances. This may have altered the post-vein path length for water. However, the distance between veins tends to equal the distance between veins and the evaporative surface in derived angiosperms (Zwieniecki and Boyce 2014). More detailed study is therefore required to determine whether the lack of variation in vein and stomatal

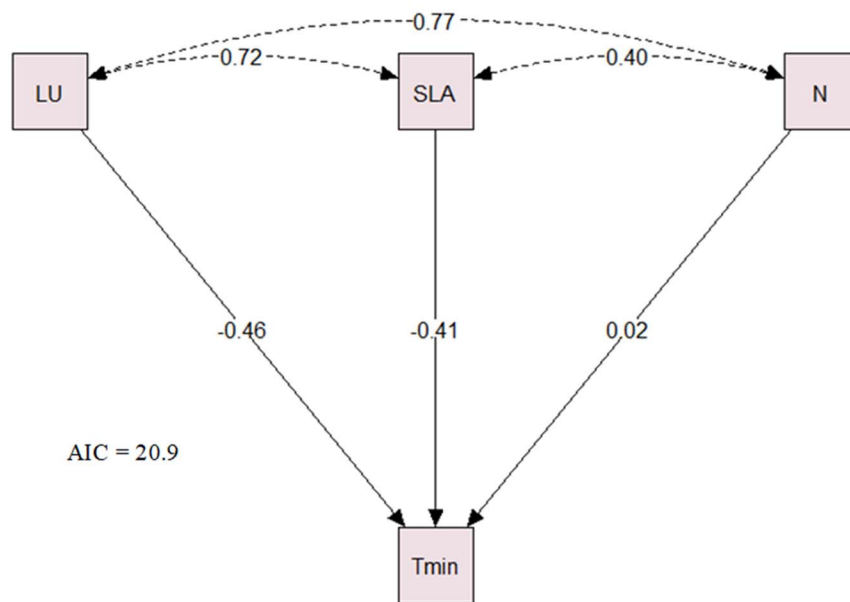


Figure 6. Path diagram of the model that best describes (lower AIC) the relationship between climate variables and functional traits. Solid and dashed lines indicate direct or indirect effects, respectively, between variables and traits. Values in the arrows indicate the standardized coefficients (std.all) and refer to standardized estimates of the variances of both continuous observed and latent variables. LU = leaf unfolding; N = leaf nitrogen content; SLA = specific leaf area; Tmin = minimum temperature.

density among provenances is due to strong plasticity or genetic changes in other leaf traits.

In many cases, traits exhibiting plasticity also show genetic differentiation (Kremer et al. 2013), unless their heritability is extremely low. Therefore, an interesting follow-up of this study would be to explore whether there is enough genetic diversity within populations on which diversifying selection may act, since it may be very important for the species potential to adapt to future changes in climate.

Increases in shoot growth and leaf area allocation due to higher temperatures result in decreases in plant N concentration and leaf N content (Weih and Karlsson 2002). This explains the negative correlation reported by the PCA between leaf N content and temperature and precipitation for the different provenances. In fact, from all the models tested to identify which climate variable determines the variance in the functional traits most influenced by climate, the one that fits best with our results suggests that minimum temperature is the main climate variable determining leaf N content, SLA and LU for each provenance.

Water-use efficiency, which corresponds to the ratio of biomass produced to the rate of transpiration, can be estimated by measuring the carbon isotope discrimination. While previous studies in oak showed intra-specific genetic variations in  $\Delta^{13}\text{C}$  in *Quercus* (*Q. robur* and *Q. petraea*, Ponton et al. 2002) (*Q. robur* and *Q. pyrenaica*, Granda et al. 2018) in *Pinus pinaster* (Lamy et al. 2014) and in *Populus nigra* (Guét et al. 2015), our results showed no significant genetic differentiation between provenances in *Q. petraea*. The lack of genetic differentiation between populations might be explained by the advantage of

increased efficiency of water use in conserving soil moisture, which could be more predominant in Mediterranean and semiarid climates. Our results, however, agree with those reported by Chamailard et al. (2011) showing no differences in leaf C content among three populations of *P. nigra* grown in a common garden, even when they were subjected to different water treatments. Whether our target species, *Q. petraea*, is less prone to the occurrence of genetic differences for given physiological traits than other species is an emerging question that needs to be addressed in future studies.

Resistance to embolism varies across plants species and helps explain species distributions across climatic gradients (i.e., water scarcity; Choat et al. 2012). However, low or no variability in resistance to embolism is usually reported within species (González-Muñoz et al. 2018, Lamy et al. 2014). Lamy et al. (2011) showed a strong uniform selection or canalization for this trait shaped the lack of genetic variation. There are some studies showing a significant correlation between  $P_{50}$  and climate variables (Brodrigg et al. 2014, Larter et al. 2017), especially temperature (Kavanagh et al. 1999), although this is not a common pattern in either 'in situ' studies or common garden experiments (Martínez-Vilalta et al. 2009, Lamy et al. 2014). Recently, Stojnić et al. (2018) attributed lack of variability to an ascertainment bias, as most populations in reported studies originate from the core of the species distribution. Hence, significant differences are observed when marginal populations growing in areas subject to unsuitable conditions for the species are considered. In our study, despite the contrasting climate at the original location of the four provenances that

would induce differences in potential evapotranspiration (no data available), xylem resistance to embolism showed similar  $P_{50}$  values. Therefore, contrary to Cavender-Bares (2018), our results do not evidence a role of evolvability for hydraulic traits that could have allowed adaptation to changing environments. The minor differences observed, however, correlate with mean temperature, with more resistant individuals originating from the warm margin. This could affect the performance of individuals from the different populations although only slightly. In fact, as for SLA, LU and leaf N content,  $P_{50}$  seems to be altered by the main climate variables evaluated (i.e., precipitation and maximum and minimum temperatures), as the PCA shows. This result is similar to what has been observed for *Quercus oleoides* in which variation among populations associated with climates of origin have been found despite a lack of local adaptation (Cavender-Bares 2018). Blackman et al. (2017) also showed how  $P_{50}$  was related to MAT across eight populations of *Corymbia calophylla* grown in a common garden experiment. Wood density has been theoretically linked with resistance to embolism based on the avoidance of implosion/collapse events in the xylem vessels (Hacke et al. 2001), because it is closely related to climate, and in particular precipitation and aridity, across species (Martínez-Cabrera et al. 2009). The lack of significant correlation between wood density and the climate variables analysed and the low contribution to the principal component 1 of the PCA (i.e., the one more influenced by climate) suggest that this anatomical trait is differently affected by climate than resistance to embolism and that, therefore, it could not work as an accurate proxy for  $P_{50}$  at an intra-specific level. Despite this, more studies including more provenances from the marginal distribution area of the species and more species with different xylem anatomical characteristics are required to definitely reject wood density as proxy for embolism resistance within species.

## Conclusions

Our results evidenced that different *Q. petraea* provenances originating from an extensive portion of the species' overall distribution in Europe showed important genetic variation for traits related to phenology and growth. In fact, genetic differentiation is much higher for leaf phenological traits than for other functional traits directly related with plant tolerance to drought, such as hydraulic traits. Most anatomical, physiological and hydraulics traits evaluated, except SLA, did not show any significant variation between provenances, or very minor genetic differentiation, although large between-tree (within populations) variations were observed. This raises the question of whether the multiple-trait associations are more driven by very integrated traits, i.e., by traits that can give synthetic information about interactions between the plant and their environment, such as growth, phenology and SLA. Despite this, there are some less integrated traits, e.g., leaf N content, highly influenced by the cli-

mate at the origin of the provenances, that although they did not show genetic divergence could be a good target of selection for individuals from marginal populations, i.e., with highly contrasted climate conditions. Whether the lack of variation in certain traits in this species is due to phenotypic plasticity remains to be investigated. In any case, we suspect that there is enough genetic variation within populations to trigger genetic divergence among populations. While experimental evolution cannot be implemented to check our hypothesis, future investigations will explore whether anatomical, physiological and hydraulics traits do exhibit significant selection gradients.

## Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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## Authors' contributions

A.K., and S.D. conceived the ideas and, together with J.M.T.-R., designed methodology. JMT-R, LT, FB, AD, and SD collected the samples and carried out most of the measurements related with phenology and anatomy. J.M.T.-R., M.R.C.M., and T.J.B. collected and analysed the vein and stomatal density data. L.J.L. and J.M.T.-R. ran the statistical analyses. All authors contributed critically to the drafts and gave final approval for publication.

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