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In situ marker-based assessment of leaf trait evolutionary potential in a marginal European beech population

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Abstract

Evolutionary processes are expected to be crucial for the adaptation of natural populations to environmental changes. In particular, the capacity of rear edge populations to evolve in response to the species limiting conditions remains a major issue that requires to address their evolutionary potential. *In situ* quantitative genetic studies based on molecular markers offer the possibility to estimate evolutionary potentials manipulating neither the environment nor the individuals on which phenotypes are measured. The goal of this study was to estimate heritability and genetic correlations of a suite of leaf functional traits involved in climate adaptation for a natural population of the tree *Fagus sylvatica*, growing at the rear edge of the species range. Using two marker-based quantitative genetics approaches, we obtained consistent and significant estimates of heritability for leaf phenological (phenology of leaf flush), morphological (mass, area, ratio mass/area) and physiological (δ^{13}C, nitrogen content) traits. Moreover, we found only one significant positive genetic correlation between leaf area and leaf mass, which likely reflected mechanical constraints. We conclude first that the studied population has considerable genetic diversity for important ecophysiological traits regarding drought adaptation and, second, that genetic correlations are not likely to impose strong genetic constraints to future population evolution. Our results bring important insights into the question of the capacity of rear edge populations to evolve.

Introduction

Characterizing the pace of adaptive evolution within natural populations is fundamental to know at which timescales evolution plays a significant role in adaptation. In particular, following abrupt environmental changes, short-term evolution, that is microevolution, may play a critical role on populations' adaptation and avoidance of extinction (Bell & Gonzalez, 2009). This issue has recently become of major concern given the anticipated climate changes that are expected to impose new selection pressures to current populations, putting them at risk of extinction (Davis *et al.*, 2005).

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Microevolutionary rates are expected to depend upon several factors among which the level of genetic variation present within natural populations might critically limit populations' capacity to respond to natural selection (Hoffmann *et al.*, 2003). Besides genetic variation, populations' evolvability will also be affected by the various evolutionary forces, selection intensity, gene flow between populations and drift, and by the genetic architecture that altogether interact with and affect genetic variation. Thus, it is now widely recognized that adaptive failures can occur despite abundant genetic variation (Futuyma, 2010). Within a given population, both selection and drift are expected to result in reduced genetic diversity over generations. On another hand, evolutionary mechanisms, such as balancing selection (Turelli & Barton, 2004; Delph & Kelly, 2014) and gene flow (Alleaume-Benharira *et al.*, 2006), can also promote adaptive genetic diversity depending on their intensity relative to directional selection and drift.

Thus, despite the apparent omnipresence of abundant genetic variation, some populations may lack genetic variation and its assessment remains a prerequisite to understand evolutionary dynamics (Hoffmann *et al.*, 2003).

Demographic and biological properties, such as generation time, fecundity rates, effective population sizes, are expected to interact with genetic diversity to determine evolutionary outcomes. In the case of long-lived species such as trees, both long juvenile phase and high longevity suggest that the amplitude of microevolutionary responses per generation must be strong in order to cope with rapid environmental changes. Trees have strong fecundity rates and large population sizes that might promote their future evolution (Petit & Hampe, 2006; Oddou-Muratorio & Davi, 2014). Moreover, tree populations have been shown to have evolved local adaptation over the last 7000–9000 years, suggesting that trees have strong evolutionary potentials (Savolainen *et al.*, 2007), although the pace of past and current climate change considerably differs in magnitude. Thus, trees harbour considerable genetic diversity between populations, leading to the idea that gene flow will be a major component of future adaptation (Kremer *et al.*, 2012). Evidences are also accumulating, suggesting that within-population genetic diversity (or standing genetic diversity) can be strong despite the strong selection (Alberto *et al.*, 2013). Considering that gene flow is spatially restricted in some tree species and thus might fail to restore adaptation (Valladares *et al.*, 2014), the question of the role of standing genetic diversity in tree populations in future adaptive dynamics remains a fundamental issue.

Moreover, gene flow from pre-adapted populations might not even be an option in some particular cases such as for marginal rear edge populations that are isolated and experience limiting climatic conditions. The capacity of rear edge populations to persist under novel environmental conditions raises the broader question of the evolutionary and ecological factors constraining current species ranges. The lack of evolutionary potential for climate adaptation is a major hypothesis, considering they are often fragmented, isolated and thus prone to drift (Hampe & Petit, 2005), but other factors such as gene swamping from neighbouring populations (Alleaume-Benharira *et al.*, 2006), genetic constraints (Etterson & Shaw, 2001) as well as interspecific competition (Stanton-Geddes *et al.*, 2012) can also explain the range limits, all these hypotheses being not mutually exclusive.

Quantitative genetics predicts that rates of phenotypic evolution depend on the narrow sense heritability h^2 (the ratio of additive genetic variances and phenotypic variances) and that the evolution of multiple traits under selection will be nonindependent if they are genetically correlated (Walsh & Blows, 2009). The classical approach to estimate evolutionary parameters is to characterize trait variation within and among categories of individuals of known relatedness (typically half-sibs families in common gardens). Common garden studies or reciprocal transplantation experiments based on progeny tests can be very powerful to assess genotypic divergence in controlled environmental conditions (Alberto *et al.*, 2013). However, due to practical reasons, such tests have some well-known limitations. First, a small number of strongly fertile mother trees are usually represented in progeny tests and thus might not be representative of the entire population. Second, in the case of trees, traits are mostly studied on young seedlings, which is relevant to understand evolution at early life stages but might not be so for later life stages. Third, the environmental conditions in common garden experiments may considerably differ from the populations native environment which can affect genetic variances and not reflect the actual variation available for selection in the wild (Conner *et al.*, 2003; Geber & Griffen, 2003).

Thus, *in situ* approaches developed within naturally established populations occurring in their native environment have the potential to bring useful insights to this issue. Comparing *in situ* estimates of genetic diversity to the genetic response of populations in common garden would shed light on the potential of tree populations to locally adapt to climate. One major issue within natural plant populations is that relatedness links among individuals are usually unknown, and moreover complex and unevenly distributed. Historically, a first solution to this problem was proposed by Ritland (1996) who developed a pedigree-free method relying on the regression of phenotypic similarities among individuals on the genetic relatedness estimated with molecular markers. This method is known to suffer from low power and bias (Coltman, 2005; Frentiu *et al.*, 2008) and its successful implementation strongly depends on the presence of highly related individuals (Ritland, 1996; Castellanos *et al.*, 2011) which can be promoted by several factors such as the mating system (selfed vs. outcrossed species), restricted seed dispersal or low effective population sizes (Vekemans & Hardy, 2004). Furthermore, the issue of spatial dependence between genetic and environmental covariances among individuals has to be addressed: depending on dispersal, highly related individuals such as full- of half-sibs may share common environment.

In the past few years, the animal model, which is an individual linear mixed model using pedigree-based relatedness matrices to model genetic variances, has benefited of great developments to account for the various statistical and biological issues typical of *in situ* studies (Kruuk *et al.*, 2008; Pemberton 2008) and has now become a very popular model for the estimation of quantitative genetic parameters within natural populations. However, for both practical and historical reasons, it has mainly been applied on animal species and

to our knowledge in only one plant study (Castellanos *et al.*, 2015). As compared to regression-based methods, linear mixed models are acknowledged to better account for the nonindependence of pairwise relatedness and thus to provide more rigorous estimations and tests of genetic variances (Frentiu *et al.*, 2008). Instead of the pedigree-based relatedness matrix, the marker-based relatedness matrix has also been used in the animal model framework. Studying a randomly mating population and a limited number of markers, Frentiu *et al.* (2008) found that regression-based approaches and the pedigree-free animal model matrix performed similarly poorly. This limitation has been recently circumvented in model species for which genomewide relatedness estimates are available (Stanton-Geddes *et al.*, 2013; Bérénos *et al.*, 2014), but to our knowledge, this approach remains to be tested in natural populations with high degree of relatedness.

This study aims at investigating the adaptive potential to drought of a marginal Mediterranean population of *Fagus sylvatica*, a species known to be particularly sensitive to summer drought (Piovesan *et al.*, 2008). In trees, water shortages are expected to induce hydraulic dysfunctions such as xylem cavitation or leaf wilting which should negatively impact tree fitness (Bréda *et al.*, 2006). Avoidance of hydraulic dysfunctions can be achieved by either reducing vulnerability to cavitation or/and by reducing water losses (through morphological or physiological traits). The within-population variability of leaf traits involved in the regulation of transpiration was not investigated in *F. sylvatica* to our knowledge. On another hand, phenological events have been shown to alter the adaptive significance of drought adaptive traits under seasonal droughts (Geber & Griffen, 2003). Although the correlated evolution of water economy and phenological trait is well established for short-lived plants, this is less clear in the case of tree species that recurrently experiment drought during their biological cycle.

Focusing on a set of six leaf functional traits related to drought adaptation, we estimated *in situ* the adaptive potential of a rear edge adult population of *F. sylvatica* for which the pedigree was unavailable. Because estimating evolutionary parameters *in situ* without a pedigree has proven to be difficult, we applied two pedigree-free approaches, that is the Ritland's approach and the animal model approach using a molecular-based (microsatellites) estimated relatedness matrix in order to get a complementary view of the adaptive potential. We asked the following questions: (i) what is the relatedness structure of the studied population, (ii) is heritability significant and does it translates into strong additive genetic variances and (iii) are the studied traits genetically correlated? Based on these questions, we first discuss of our ability to estimate the evolutionary potential of the studied population. Second, we discuss about the evolutionary potential of the studied population to drought stress. Finally, considering that this particular population might have a low evolutionary potential as compared to the main populations, we discuss about the implications of this study regarding the evolutionary potential of tree populations.

Materials and methods

Studied population

European beech (*F. sylvatica,* Fagaceae) has a large continental distribution from the northern part of the Mediterranean region to the South of Sweden. It is a monoecious, anemophilous, mainly outcrossing species. Individuals typically begin to reproduce after 40–50 years, and seeds are produced in irregular mast years. As for many tree species (Austerlitz *et al.*, 2004), we found in a previous study conducted on Mont Ventoux (in other parts of the population than studied here) that some long-distance dispersal events may occur (in particular for the pollen); however, most pollen grains and especially seeds are dispersed over relatively short distances, with the average distance of seed dispersal ranging between 11.9 and 21.7 m and the average distance of pollen dispersal ranging between 15.8 and 48.2 m (Bontemps *et al.*, 2013). European Beech is a shade-tolerant species requiring high atmospheric humidity due to a weak tolerance to summer drought.

The study site is located on the Mont Ventoux (South-Eastern France, 44°10′28″N; 5°16′16″E). The Mont Ventoux population belongs to a broader ensemble of scattered and disjoint populations typical of range margins with the nearest population occurring on the Lure mountain located at ~90 km away. European beech recently recolonized the slopes of Mount Ventoux under the black pines (*Pinus nigra*) which were planted at the end of the 19th century as part of a massive national afforestation programme (Lander *et al.*, 2011). In Mont Ventoux, European beech ranges from ~900 m (occasionally 750 m) to ~1700 m in elevation, covering an approximately 90 ha surface. In this study, we focused on an elevation range of 987–1048 m. Therefore, the focal population was at the lower limit of the local species' distribution. Regarding the ecological conditions on Mont Ventoux, the bedrock is calcareous with highly variable soil depth and high content in coarse elements (Nourtier *et al.*, 2014). The climate varies along elevation, but in the study site it is Mediterranean, that is characterized by strong summer droughts, with rainfalls mostly occurring in spring and autumn (Cailleret & Davi, 2011). Finally, in the study site, beech is the dominant species, but it is mixed with other tree species and with silver fir (*Abies alba*) in particular.

In Mont Ventoux, beech is often found in clusters of multiple clonal stems due to root suckering or resprouting (favoured by coppice management). In the study plot, ~75% of beech trees were found in such clusters, probably due to the former intensive exploitation of beech wood for firewood production.

Sampling scheme

The study plot has an area of 0.83 ha where we exhaustively mapped 149 reproductive adult trees and measured their diameter at 1.3 m (dbh). A tree was considered to be reproductive when its dbh was > 12 cm. In the case of clusters, only the biggest stem (generally dominant) of the cluster was sampled for trait measurement. Additionally, we checked for the presence of clones among visually defined clusters using genetic analysis (see Bontemps *et al.*, 2013 for details) and kept only the biggest individual in case of clones (25 clones detected).

Measurement of leaf functional traits

Three main categories of phenotypic traits were recorded.

(1) Phenological traits: the *in situ* dynamic of leaf flush was recorded for the 145 adult trees by monitoring leaf unfolding stages twice a week 1/for the year 2009, from the 23rd of March to the 4th of May and 2/for the year 2010, from the 8th of April to the 10th of May. Five stages corresponding to five scores (from 1 to 5) were defined following the standard international code (BBCH code, Finn *et al.*, 2007). We summed daily scores to compute a phenology score sum (PSS) for each tree. Higher is the PSS, earlier and quicker is the process of leaf unfolding.

(2) Morphological traits: they were recorded for the same trees than the phenological traits. In July 2009, we collected three samples of ten terminal leaves per tree, at the top of the crown. Traits measured were as follows: leaf dry mass (LM), leaf area (LA), leaf mass per area (LMA) computed as the ratio of LM on LA and leaf water content (W%) computed as the ratio of wet mass minus dry mass on wet mass.

(3) Physiological traits: out of the three sets of ten leaves collected per individual, the one having the highest LMA value was used for subsequent physiological analyses: leaf carbon isotope discrimination ($\delta^{13}C$) and leaf nitrogen content (N_{mass}). Carbon isotopes and nitrogen analyses were performed with a continuous flow isotope mass spectrometer coupled to an elemental analyser (Roussel *et al.*, 2009) at the Technical Platform Functional Ecology Research Centre INRA Nancy (PTEF, web link).

Genotyping & Quality assessment

Additionally to previous samples, two leaves were collected on each tree and stored at −20 °C. We genotyped them with 19 microsatellite markers in three multiplexes (numbers in parentheses indicate the multiplex): (i) FS1-15, FS3-04 (Pastorelli *et al.*, 2003); sfc0007-2, sfc0018, sfc0036, sfc1063, sfc1143, (ii) sfc0161 (Asuka *et al.*, 2004); csolfagus-7, csolfagus-10, csolfagus-13, csolfagus-19, Fi-05, (iii) csolfagus-5, csolfagus-6, csolfagus-25, csolfagus-29, csolfagus-31, (Vendramin, pers.comm.); mfc7 (Tanaka *et al.*, 1999). Total DNA was extracted from 50-mg-wet-weight frozen leaf material using the protocol for the DNeasy 96 Plant kit (Qiagen, Hilden, Germany). The concentration and purity of the DNA were estimated by measuring the absorbance at 260 and 280 nm in a spectrophotometer and using pulse-field gel electrophoresis on agarose gel. We used the original PCR conditions given by the authors. PCR products were separated on an automated 96-capillary MegaBACE™ 1000 sequencer (GE Healthcare, Little Chalfont, UK). Genotypes were sized using the internal size standards ET400 and the MegaBACE™ FRAGMENT PROFILER ver. 1.2 software (GE Healthcare). Automatic allele assignment was checked and revised visually twice to ensure consistency of genotyping.

Population genetic parameters and test of the F_{is} significance was performed with FSTAT (Goudet, 1995). The loci were also tested for linkage disequilibrium in FSTAT. Null allelic frequencies (NAF) per locus were estimated using the ML-NULL software (Kalinowski & Taper, 2006).

Estimation of relatedness coefficients

We computed pairwise relatedness coefficients r_{ij} among the 145 adult trees using the 19 microsatellite markers. Here, we consider the definition of the relatedness coefficient (r_{ij}) as the expected fraction of alleles in the genome that two (related) individuals have identical by descent (Van de Casteele *et al.*, 2001). Two method-of-moment relatedness estimates (r_{ij}) were used: the Lynch & Ritland (1999) [LR] and the Wang (2002) [WG]. We also computed the so-called actual variance of relatedness Vr_{ij} using the estimator described in Ritland (1996), which separates the variance component due to the random segregation of alleles from the total variance of the molecular marker-based estimate of r_{ij} ($V_{tot}r_{ij}$). Allelic frequencies were estimated from the sampled population. The LR and WG estimators have different properties in terms of bias and precision that depends on the populations relatedness structure (Van de Casteele *et al.*, 2001). We compared the performance of each estimator for the estimation of heritability using Ritland's method. We found that overall, LR-derived estimates of heritability were more precise, in particular when the proportion

of unrelated individual was high. In the following, we will only consider the results obtained using the LR estimator, results with the WG estimator given as (Fig. S1).

Spatial analysis of relatedness

The classical analysis of the spatial distribution of relatives consists of plotting the variation in average genetic relatedness among pairs of individuals (r_{ij}) against their distance (or logarithm of distance in a two-dimensional space). Pairwise relatedness were computed using two estimators of relatedness, (i) developed by [Lynch and Ritland (1999), LR] and (ii) developed by [Wang (2002), WG]. In order to visualize the spatial structure of relatedness, we plotted the average relatedness per distance class $r_{ij}(d)$ using 5-m steps. Moreover, we reported both the $r_{ij}(d)$ as well as the variance in relatedness Vr_{ij} (d) for the first distance class. Standard errors around $r_{ij}(d)$ were obtained with jackknife procedures that consisted of deleting one locus at a time. To test spatial genetic structure the $r_{ij}(d)$ values were regressed on $\ln(d_{ij})$, where d_{ij} is the spatial distance between individuals i and j, to provide the regression slope b. Then the spatial positions of the individuals were permuted 5000 times in order to get the distribution of b_{log} values expected under the null hypothesis that $r_{ij}(d)$ and d_{ij} were uncorrelated. All genetic calculations were performed using Spagedi (Hardy & Vekemans, 2002).

Estimation of the genetic parameters: the Ritland's regression approach

Heritability

For all studied functional traits, the phenotypic similarity between a pair of individuals i and j (Z_{ij}) was regressed against their relatedness (r_{ij}) estimated using molecular markers.

Z_{ij} is computed as follows:

$$Z_{ij} = \frac{(P_i - \bar{P})(P_j - \bar{P})}{\mathrm{var}(P)} \quad (1)$$

where P_i, P_j, the phenotypic values of individuals i and j, respectively.

In a uniform environment, Z_{ij} writes as a function of h^2, the narrow sense heritability, and a_e, the intercept:

Model 1:

$$Z_{ij} = h^2 r_{ij} + a_e + \varepsilon_{ij} \quad (2)$$

where ε_{ij} is the random error.

However, in highly heterogeneous environment, the distance between individuals may also affect their environmental correlation, which can inflate the estimate of h^2 in case of spatial clustering of relatives, the environmental covariance being confounded with the genetic covariance. To control for this effect, Ritland (1996) introduced within eqn 2. the parameter b_e which accounts for the decrease in environmental correlation with distance between individuals:

Model 2:

$$Z_{ij} = h^2 r_{ij} + b_e d_{ij} + a_e + \varepsilon_{ij} \quad (3)$$

where d_{ij} is the Euclidean spatial distance.

Genetic correlations

The genetic correlations are estimated through the additive genetic covariances scaled by the root square of the product of the additive genetic variances of both traits. Following Ritland's model (1996):

$$\rho(i,j,A,B) = \frac{\mathrm{cov}\left((p_{Ai} - \mu_A)(p_{Bj} - \mu_B), r_{ij}\right)}{\sqrt{\mathrm{cov}\left((p_{Ai} - \mu_A)(p_{Aj} - \mu_A), r_{ij}\right)\mathrm{cov}\left((p_{Bi} - \mu_B)(p_{Bj} - \mu_B), r_{ij}\right)}} \quad (4)$$

with p_{Ai}, p_{Bi}, p_{Aj}, p_{Bj} the phenotypes for traits A and B of individuals i and j, respectively, and μ_A and μ_B the mean of trait A and B.

Spatial analyses

Following Ritland's recommendations, we defined a set of cut-off distances ranging from 5 to 100 using 5-m steps. For each cut-off distance, we estimated both the heritability and the genetic correlations for all the studied traits (listed in Table 1), keeping only the pairs of individuals having a Euclidean distance inferior to the cut-off distance. In the result section, we refer to the heritability coefficient using the classical abbreviation h^2 followed by the cut-off distance and finally by the trait abbreviation, ex: $h^2$30-PSS is the heritability estimated for budburst phenology using the 30 m cut-off distance.

The impact of the spatial scale on the analysis is threefold. First, at short distances, we expect to get a better balance between related and unrelated pairs of individuals, and therefore a higher variance in relatedness, than at longer distance where related pairs are rare (Ritland, 1996). Second, at short distance, we expect less confounding effect between genetic and environmental covariance between paired individuals than when pairs of individuals at short and long distance are bulked together. Third, contrasting with the previous effects that tend to increase the statistical power at short distance, restricting the analysis at short cut-off distances might reduce its statistical power due to the variation of the regression parameter h^2 from place to place depending on local environments (while assuming a single value for large spatial scales).

Table 1 Variation of studied functional and performance traits and of traits used as covariates in the analyses. Unit is given in parenthesis in the 'trait' column. *N* gives the number of adult individuals successfully phenotyped (among the 149 targeted).

Trait	Abbreviation	*N*	Mean	Standard Deviation
Leaf unfolding phenology*	PSS	146	46.88	5.0
LMA ($g.m^2$)	LMA	142	98.65	14.99
Leaf area (cm^2)	LA	142	8.59	2.7
Leaf mass (mg)	LM	142	85.49	30.84
Nitrogen content	N_{mass}	142	2.22	0.28
Leaf $\delta^{13}C$	$\delta^{13}C$	142	−27.30	0.78

*measured as the Phenology Score Sum, without unit, see Materials and methods.

Test of statistical significance

Both for the heritability and the genetic correlations, we performed nonparametric bootstraps in order to compute the 95% confidence interval around the estimated parameters. (i) We resampled 150 individuals from the initial sample, either trait by trait for heritability or by pairs of traits for the genetic correlations, (ii) we estimated the heritability or the genetic correlations, and (iii) we repeated this procedure 10 000 times. The resulting distribution was used to compute the 95% confidence intervals. These computations were performed using the 'boot' function implemented in the statistical software R (R Core Team, 2015).

Moreover for heritability, we tested the null hypothesis that there were no genetic and spatial effects on the phenotype by permuting 5000 times without replacement the phenotypes across individuals while holding constant both their spatial positions and genotypes. This method advantageously maintains the covariance of relatedness and spatial distance, the actual variance of relatedness and the variance of spatial distance constant (Andrew *et al.*, 2005). However, this also removes the covariance of phenotypic similarity with distance. In case of shared environments between relatives, we would be overconfident on the estimations made with model 1, whereas the estimations made with model 2 should be correctly tested (see Andrew *et al.*, 2005 for more details).

Estimation of the genetic parameters: the Bayesian animal model approach

We estimated the additive genetic covariance matrix *G*, of the studied functional traits using the R package MCMCGLMM (Hadfield 2010) that implements the estimation of linear mixed models in a Bayesian-MCMC framework. We set the six studied functional traits, that is LMA, LA, LM, PSS, $\delta^{13}C$ and N_{mass} as response variables, the traits phenotypic mean as fixed effects, the individual additive genetic merit as random effects and we specified that the traits had a normal distribution. The estimation of the additive genetic covariance matrix using an animal model requires a pedigree-based relatedness matrix. In our study, we used a marker-based relatedness matrix in place of the pedigree-based relatedness matrix.

The specification of priors for the probability distribution of the covariance matrix is a key step in using MCMCGLMM as it may influence the outcome of the posterior probability distribution and especially so when the data are poorly informative. Thus, it is strongly recommended to explore various priors. Assuming that both the genetic and residual variances were sampled from inverse Wishart distributions, we used as parameters of the priors: the identity matrix multiplied by 0.5 Vp, 0.3 Vp or 0.1 Vp and d.f. of 6, which is the dimension of the matrix to be estimated (Hadfield 2010, Stinchombe *et al.* 2014). Other parameters for the Wishart, such as lower d.f. and lower variances on the diagonal, lead to computational failures. For all models, we used 10 000 burn-in-iterations followed by 200 000 iterations and a thinning interval of 100 in order to obtain 2000 samples of the estimated parameters.

We monitored autocorrelations among posterior estimates for each component of variance–covariance in order to assess the quality of the runs. We determined the most adequate set of prior by comparing the models on the basis of their deviance information criterion (DIC), the one with the lowest DIC being preferred. Point estimates of additive genetic variances (σA), environmental variances (σE) and heritability [$\sigma A/(\sigma A+\sigma E)$] were obtained by computing their mean values over the 2000 MCMC samples. Uncertainty for the point estimates was measured as the 95% highest posterior density (HPD) for σA, σE and heritability.

Results

Microsatellite diversity

The number of alleles ranged from 4 to 16 per locus with an average of 8.42 alleles per locus (Table S1), which gives a total number of 160 alleles. This value falls within the range of previous studies that performed marker-based estimation of heritability (113 total number of alleles in Andrew *et al.*, 2005). The observed heterozygosity H_o ranged from 0.36 to 0.88 with on average $H_o = 0.67$. Using the Bonferroni correction of the significance threshold, we detected no significant linkage between loci nor significant *Fis* values. Overall, loci suffered low null allelic frequencies ($NAF_{mean} = 0.017$), but two loci were more prone to high NAF: sfc0018 (NAF = 0.087) and csolfagus-10 (NAF = 0.104).

Spatial variation of relatedness

The studied population displayed a strong and significant spatial genetic structure [LR: $b_{log} = -0.033$

(−0.030; −0.038), WG: $b_{log} = -0.043$ (−0.038; −0.048)] that decreased sharply until ~25 m, where individuals were no longer more related than expected by chance (Fig. 1). Both mean and variance of relatedness were high at short distances [LR: r_{ij} (5 m) = 0.19, Vr_{ij} (5 m) = 0.071, WG: r_{ij} (5 m) = 0.23, Vr_{ij} (5 m) = 0.089] suggesting the mixed presence of highly related and unrelated individuals. For example, the mean relatedness in the first class of distance [r_{ij} (5 m)] was very close to the expected relatedness of half-sibs (0.25) and the variance in relatedness [Vr_{ij} (5 m)] was comparable to the variance of a population composed by ½ of full-sibs and ½ of unrelated individuals (~0.06).

Heritability and genetic correlations of leaf functional traits

Ritland's regression approach

Hereafter, we present the results of model 2 that includes both the heritability and a spatial parameter, as we expect they suffer less bias and are more adequately tested (Ritland, 1996; Andrew *et al*., 2005), even though we found that distance modified the heritability estimate for LMA only (Table S2).

Heritability. Four traits had significantly non-null heritability estimates: budburst phenology, carbon isotope discrimination, nitrogen content and leaf mass (Fig. 2, Table 2). Budburst phenology overall exhibited very high levels of heritability ($h^2$30-PSS = 0.84, $h^2$100-PSS = 0.92). Nitrogen content and leaf mass were also found to be highly heritable ($h^2$30-N_{mass} = 0.47, $h^2$100-N_{mass} = 0.82, $h^2$30-LM = 0.50, $h^2$100-LM = 0.93). Leaf carbon isotope discrimination exhibited intermediate level of heritability that overlapped the significance threshold ($h^2$30-δ^{13}C = 0.37, $h^2$100-δ^{13}C = 0.52). Leaf area was found to have nonsignificant h^2 for short cut-off distances (< 40 m), but h^2 became significant for larger cut-off distance (Fig. 2). Leaf mass per area was found to be nonheritable (Fig. 2). Finally, comparing the estimates obtained using either Vr_{ij} or $V_{tot}r_{ij}$, revealed that the previous were overall higher than the latter and especially for large cut-off distances (Fig. 2a vs. Fig. 2b).

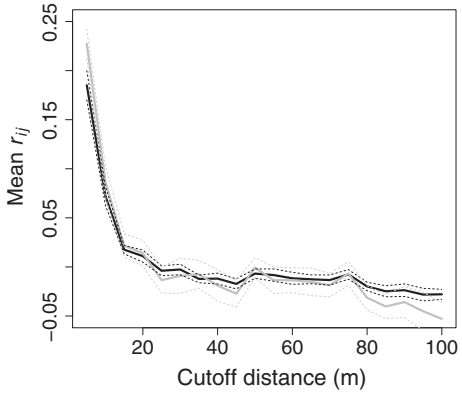

Fig. 1 Spatial genetic structure of the studied population. The relatedness coefficient r_{ij} between pairs of individuals i and j was estimated using 1) the Lynch & Ritland estimator (black line), and the Wang estimator (grey line). Average relatedness per distance class r_{ij} (d) were plotted using 5-m steps. Dotted lines are the 95% confidence intervals.

Genetic correlations. Leaf mass and LA were found to be significantly and strongly positively correlated (Fig. 3). Other pairs of traits were only correlated at some particular cut-off distances. In particular, for cut-off distances over 50 m, we found a significant positive correlation between leaf mass and leaf nitrogen content, as well as between LA and leaf nitrogen content (Fig. 3). Furthermore, for cut-off distances over 40 m, budburst phenology was negatively correlated both with nitrogen content and leaf mass; that is, early bursting genotypes had lighter leaves and lower nitrogen content.

Bayesian animal model approach

The posterior appeared to be slightly sensitive to the specified prior, the estimated heritability becoming slightly higher as the prior variance increased (about 10% for all traits between 0.1Vp and 0.5Vp, Table 3). The following results correspond to the model with the lowest DIC, which corresponded to the following parameters: 0.1Vp and 6 degrees of freedom for the Wishart distribution (Table 3). The autocorrelation values among the posterior samples for the components of variance–covariance were all less than 0.10 indicating that the quality of the analysis was acceptable (Hadfield 2010).

Heritability. Bayesian analyses suggested that all the studied functional traits had moderate heritabilities, with posterior means ranging from 0.20 to 0.31 (Table 3). These results were generally lower than the values obtained using the Ritland's model after correction of the variance of relatedness (Fig. 2a). This was less the case when comparing Bayesian estimates with Ritlands estimates without correction of variance of relatedness, and in particular, both estimates converged with large cut-off distance (sample including ~all pairwise comparisons, Fig. 2b). The ranking of traits for their heritability estimates was broadly conserved among the two types of analyses, although in both of them, traits confidence intervals greatly overlapped in most cases. One noticeable difference was that we estimated a significant but low heritability for LMA with the Bayesian approach and not with Ritland's method. Unlike in Ritland's analyses, we did not control for spatial effects in the Bayesian analyses.

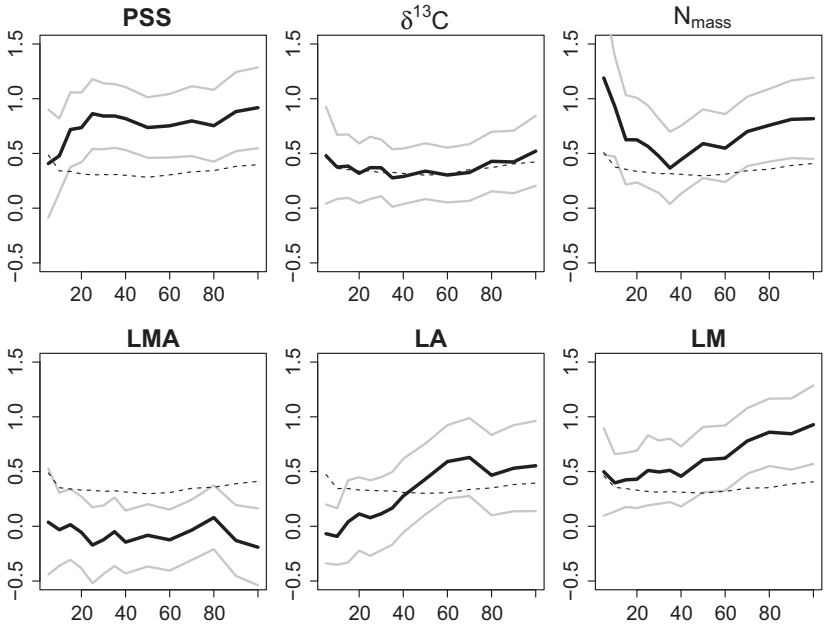

Fig. 2 Heritability estimates using the Ritland's regression method as a function of the pairwise cut-off distances (in metres, traits and corresponding abbreviation listed in Table 1). Computations were performed using the actual variance of relatedness Vr_{ij} (see section 'Estimation of relatedness coefficients' in Materials and methods). Point estimates are represented by the black line, the 95% CI interval (obtained using bootstraps, see section 'Test of statistical significance' in Materials and methods) by the grey lines and the null hypothesis 95% threshold (obtained using randomization, see section 'Test of statistical significance' in Materials and methods) by the dotted black line.

Table 2 Heritability coefficients for the studied functional trait using the Ritland's linear regression method. The models presented here were estimated using either the corrected or the noncorrected variance of relatedness (Vr_{ij} and $V_{tot}r_{ij}$, see Materials and methods). For each of these two model categories, we presented the models performed using a 30- or a 100-m cut-off distance. Point estimates of traits' heritability (bold letters) and lower and upper bounds of the 95% confidence intervals (following first and second rows) estimated using Ritland's method. Estimates were obtained using both the corrected (Vrij) and the total variance of relatedness (Vtotrij) for two cut-off distances (30m and 100m)

Var (r_{ij})	Cut-off	PSS	δ^{13}C	N_{mass}	LMA	LM	LA
With correction	30 m	**0.84**	**0.37**	**0.47**	**−0.12**	**0.50**	**0.11**
		0.54	0.11	0.14	−0.44	0.21	−0.22
		1.14	0.63	0.82	0.19	0.78	0.45
	100 m	**0.92**	**0.52**	**0.82**	**−0.19**	**0.93**	**0.55**
		0.55	0.20	0.45	−0.54	0.57	0.14
		1.29	0.84	1.19	0.16	1.29	0.96
Without correction	30	**0.65**	**0.28**	**0.40**	**−0.09**	**0.38**	**0.26**
		0.44	0.08	0.15	−0.33	0.17	0.07
		0.86	0.48	0.66	0.15	0.60	0.45
	100	**0.36**	**0.22**	**0.38**	**−0.08**	**0.40**	**0.27**
		0.22	0.09	0.22	−0.23	0.25	0.13
		0.51	0.36	0.53	0.07	0.55	0.41

Genetic correlations. Regarding the genetic correlations, similar to the Ritland's analysis, we found a strong positive genetic correlation between leaf mass and LA [ρ = 0.75 (0.43–0.91), Fig. 4]. All the remaining genetic correlations were not significant. However, some tendencies were found, indicating some similarities with the Ritland's analysis regarding the correlations between the physiological and morphological traits. In particular, we found that over > 90% of the MCMC estimates were positive regarding the correlation Nmass/LA. It seems interesting to observe a difference between the posterior and the prior considering that our data are *a priori* poorly informative.

Discussion

Estimating *in situ* heritability and genetic correlations in trees

In situ estimation of the evolutionary potential is an important step for understanding the evolutionary dynamics of natural populations (Kruuk *et al.*, 2008). In this study, we detected significant amounts of additive genetic variance for the studied leaf functional traits. We had 19 microsatellite markers available representing 160 alleles in total for the estimation of the relatedness matrix. This can be considered already as an important genotyping resource for a nonmodel species. However, this is comparable to those used by previous studies that found both imprecise and downwardly biased heritability (Coltman, 2005; Frentiu *et al.*, 2008). One successful way to avoid these problems has been to increase the number of markers genomewide (Stanton-Geddes *et al.*, 2013; Bérénos *et al.*, 2014). Apart from the number of markers, the relatedness structure jointly with the sample size can also greatly affect the precision of heritability estimates (Ritland, 1996). Hence, we were able to achieve good statistical power probably because of the high proportion of highly related

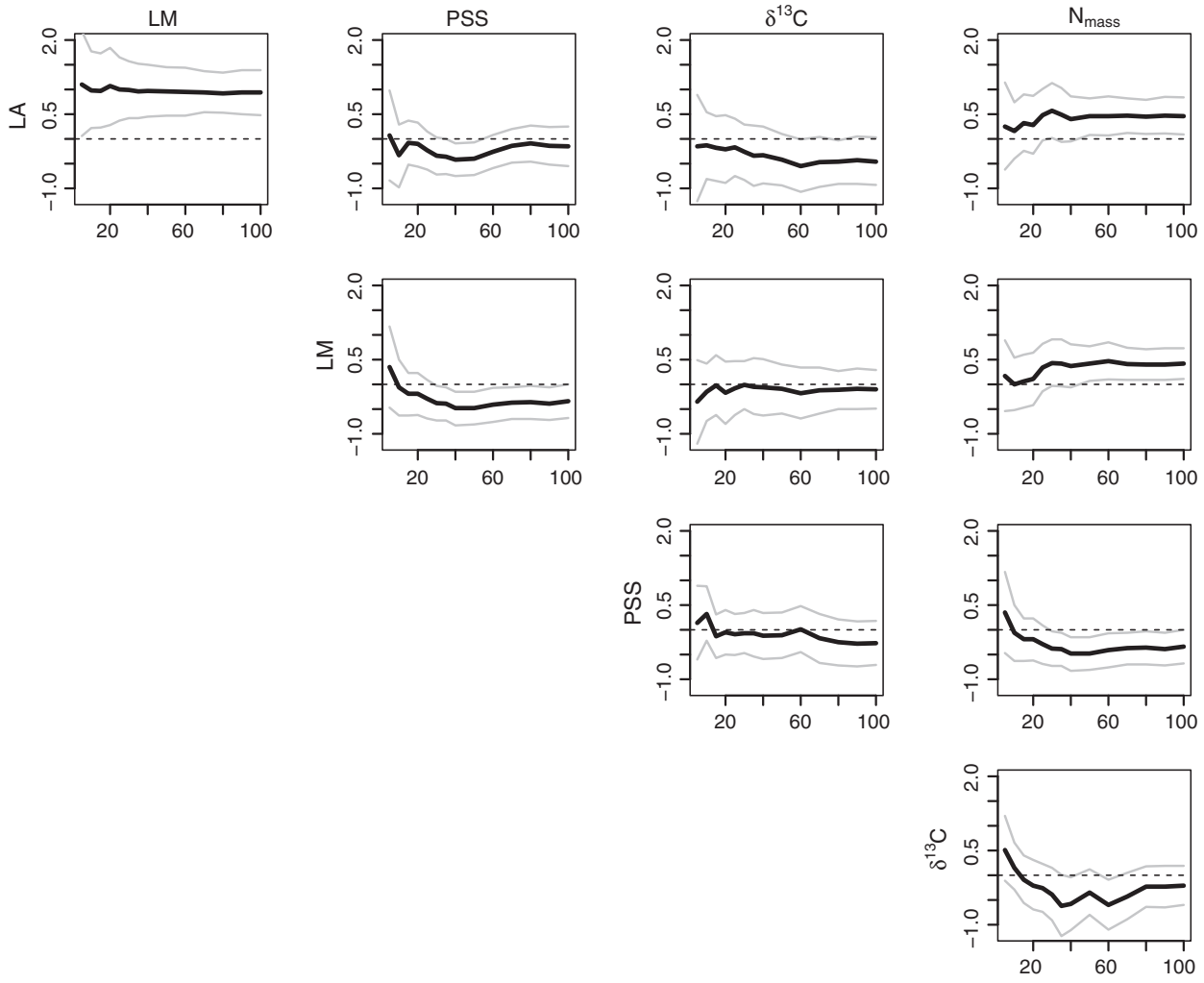

Fig. 3 Genetic correlation estimates using the Ritland's regression method as a function of the pairwise cut-off distances (in metres, traits and corresponding abbreviations listed in Tab 1). Point estimates are represented by the black line, the 95% CI interval (obtained using bootstraps, see section 'Test of statistical significance' in Materials and methods) by the grey lines. Dotted line indicates zero-value.

individuals (strong phenotypic covariance) intermixed with unrelated individuals (no phenotypic covariance) as evidenced by the strong spatial genetic structure. Strong spatial genetic structures are often observed in tree species that tend to disperse both seeds and pollen at short distances (Austerlitz *et al.*, 2004; Vekemans & Hardy, 2004;). Moreover, the demographic history with a parental population likely represented by a few founders (Lander *et al.*, 2011) has very likely contributed to patches of highly related individuals.

On another hand, this spatial aggregation of relatives can lead to overestimations of genetic parameters if the scale of dispersal is confounded with the scale of spatial heterogeneity (Ritland, 1996). Excessive correction for spatial environmental covariance between individuals in Ritland's model 2 might explain the nonsignificant heritability estimate for leaf mass area, which was significant with the Bayesian approach, although the high sensitivity to spatial effects also suggests that LMA is poorly heritable. Moreover, maternal effects are another classical source of overestimations (Kruuk *et al.*, 2008). In this study, it was impossible to account for it. However, in a companion study, the same traits have been studied on seedlings from the same population and no maternal effects were found after the second year of growth in the common garden (Gaüzère, 2014). Moreover, considering the age of the study tree, maternal effects if there were present, were unlikely to persist.

In order to get a better view of the evolutionary potential of the studied population, we compared the results from two pedigree-free methods, the Ritland method and the animal model. The animal model is usually assumed to produce more conservative results

Table 3 Heritability coefficients for the studied functional trait estimated using the Bayesian animal model (Hadfield 2010). The models presented here were estimated using as a prior variance, 10%, 30% and 50% of the total observed phenotypic variance successively (%VP, see Materials and methods). DIC is the deviance information criterion. In the following, columns are the traits' heritability statistics, with in bold, the point estimate of traits' heritability and in the following first and second line, the lower and upper bounds of the 95% confidence interval, respectively (Traits and corresponding abbreviation listed in table 1).

%VP	DIC	PSS	$\delta^{13}C$	N_{mass}	LMA	LA	LM
10	3346.5	**0.31**	**0.22**	**0.29**	**0.20**	**0.23**	**0.25**
		0.11	0.06	0.11	0.07	0.07	0.08
		0.52	0.38	0.47	0.37	0.39	0.43
30	3362.8	**0.39**	**0.30**	**0.37**	**0.29**	**0.31**	**0.32**
		0.20	0.15	0.21	0.14	0.15	0.16
		0.59	0.47	0.54	0.44	0.48	0.49
50	3380.6	**0.41**	**0.34**	**0.38**	**0.32**	**0.34**	**0.35**
		0.22	0.18	0.23	0.17	0.18	0.18
		0.59	0.50	0.54	0.48	0.49	0.52

(Frentiu *et al.*, 2008), which was in general the case of this study. Thus, the estimates were generally lower when using the animal model, one likely reason coming from the strong statistical noise present in our data set. In fact, the observed differences are compatible with the strong confidence intervals of heritability estimates together with the slight sensitivity of Bayesian models to the priors. Regarding the question of the evolutionary potential of the studied population, we suggest that finding comparable results among the two methods advocates for a substantial genetic control of the studied functional traits.

In situ heritability of the leaf functional traits

With this study, we showed within a rear edge population that the genetic basis of the phenotypic variation of ecologically important traits was substantial. Considering that the studied population experiments spatially heterogeneous water resources due to fine-scale soil variations (Nourtier *et al.*, 2014), we can expect strong environmental variances for the studied traits. Thus, detecting a significant genetic basis suggests that the genetic diversity of the studied population is strong enough to be detected despite the environmental variance. Overall, our study suggests that the lack of genetic variation is not a strong constraint for the evolution of the studied population.

First, regarding budburst phenology, if we extrapolate the estimated h^2 for PSS to the date of passage between stages 2 and 3 in the BBCH phenological scale, 95% of the breeding values ranged over 3–5 days. The range of genetic variation reported here within a single population is of the same order of magnitude (~4 days) as the range of genetic differentiation between the extreme altitudinal beech populations in SW France (Pyrenees Mountain) reported by Vitasse *et al.* (2010) and about a third, half of the 10 days between continental scale provenances (Robson *et al.*, 2013;). Although these values should be compared with caution because of differences in climates among the studies, this shows strong genetic variation for budburst phenology. Budburst phenology is usually viewed as a highly plastic trait. Several studies looking at wide environmental ranges found much greater phenotypic variation due to plasticity than to genetic diversity (Vitasse *et al.*, 2010; Oddou-Muratorio & Davi, 2014). The main environmental drivers of budburst variation (temperature and photoperiod) usually vary at larger spatial scales than considered here, which is likely the reason why we were able to detect a significant genetic basis for this trait.

The heritability estimate found for leaf carbon isotopic discrimination considered together with the considerable range of phenotypic values reveals that the additive genetic variance within the studied population is quite high. Similarly, in cork oak, Ramirez-Valiente *et al.* (2010) found significant within-population heritability estimate for this trait ($h^2 = 0.47$) for seedlings grown in a common garden experiment. Leaf carbon isotopic discrimination is classically used as a proxy of water use efficiency (WUE), which is an important trait for adaptation to water stress, with higher WUE being expected to adaptive in dry environments. However, the adaptive value of this trait depends on a complex balance of various factors and this balance will depend upon the intensity of drought and seasonality of rainfall patterns but this remains to be more deeply investigated in tree species. Existing studies performed on trees, and on Fagaceae in particular, had difficulties in confirming the existence of a genetic response for higher WUE of populations undergoing drought (Ramirez-Valiente *et al.*, 2010) and other differentiation patterns have even been occasionally observed (Brendel 2012).

Regarding the morphological traits, we found a significant genetic diversity for LA, whereas, for LMA, we obtained divergent results with different methods. The literature provides a wealth of evidences that both small LA and small LMA are key traits for the adaptation to xeric conditions (Geber & Griffen, 2003). In *F. sylvatica*, existing studies suggest that both traits respond to drought (Bussotti *et al.*, 2005). The mean values of LA and LMA in our study site were comparable to the lowest value found by Bussotti *et al.*, 2005 that concerns a Calabrian population (Italy) growing in extreme xeric conditions. Hence, we expect that the evolution of the leaf morphological traits, and in particular of LA, is a key component of adaptation to current and future droughts within the studied population.

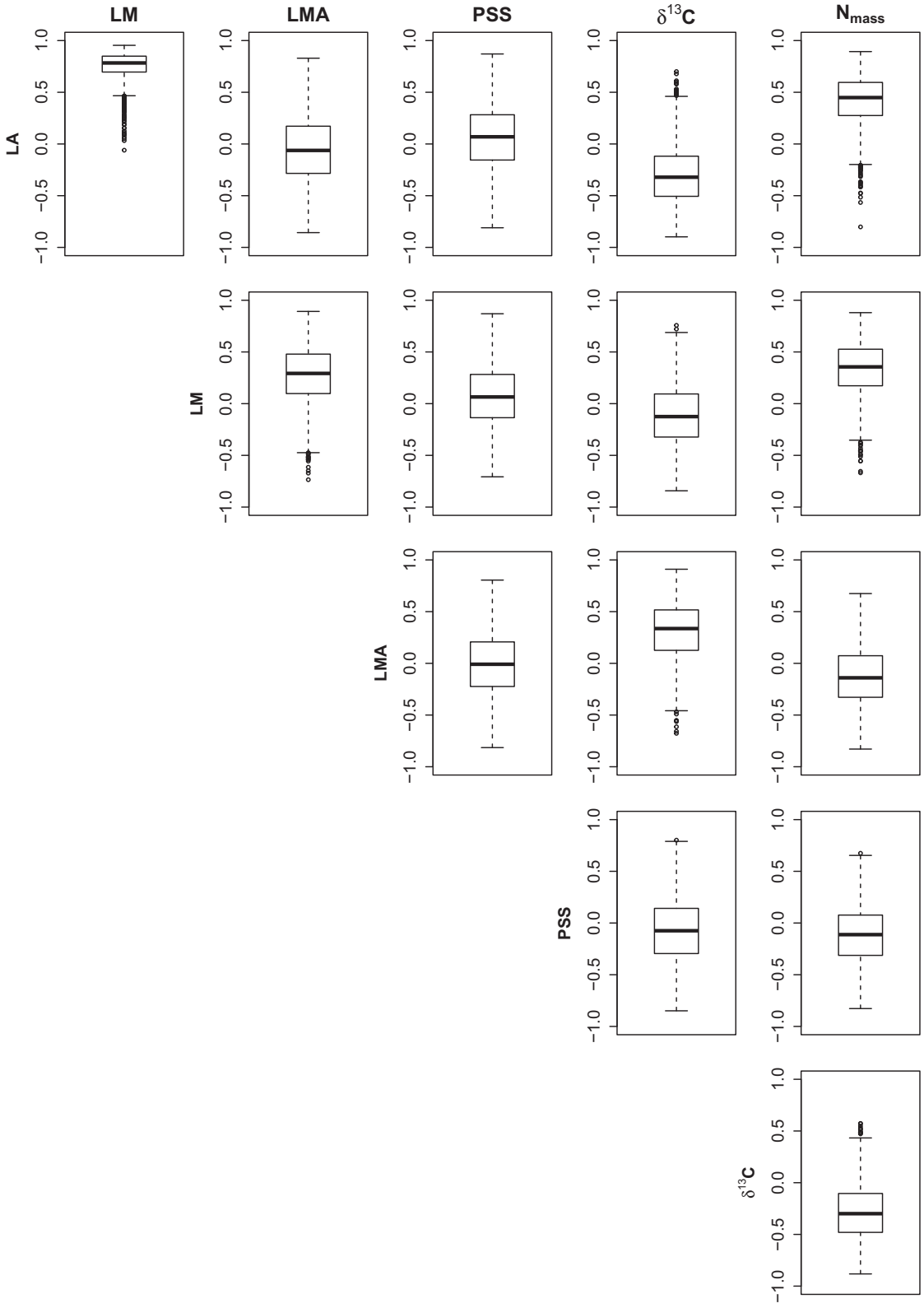

Fig. 4 Genetic correlations estimated using the Bayesian linear mixed model: boxplots of the 2000 MCMC samples (Traits and corresponding abbreviation listed in table 1).

In situ genetic correlations of the leaf functional traits

In a multivariate perspective of trait evolution, it is important to assess the genetic correlations between traits because they affect the amount of genetic variation available for multivariate selection and can thus influence the speed of evolution (Walsh & Blows, 2009). The impact of the genetic correlations will depend on their sign and magnitude. Thus, although it is notoriously difficult to get precise estimates of the genetic correlations, it is worth assessing those that are the most likely to affect multivariate evolution. We detected a strong positive correlation between leaf mass and LA using both the Ritland's and the Bayesian methods. This strong correlation is likely due to physical constrains, the big leaves being mechanically heavier that the small leaves. Regarding its impact on future evolution, this correlation is difficult to interpret. Usually, it is the relative adjustment of mass relatively to area, that is LMA that is considered relevant for adaptation to drought. Yet, we detected no sign of genetic correlation between LA and LMA and we thus assume that these traits will evolve independently in the studied population. This might not be the case for the evolution of the physiological traits for which our results suggested that some of them might be genetically correlated. However, more work is needed to unambiguously assess this issue within the studied population.

Evolutionary potential of the studied population

Finding substantial levels of additive genetic variances in this population is of particular interest because we would expect it to have lower diversity than other populations in the distribution range of the species. First, it is a typical marginal rear edge population that is remotely located from the main core and is thus expected to be genetically isolated (Hampe & Petit, 2005). Moreover, in the studied site, the population experiments harsh conditions regarding the species' requirements in terms of summer droughts, which is expected to result into both strong directional selection and strong drift, particularly in this area where overexploitation has occurred in the last centuries, which was supported by the observed strong spatial genetic structure. In a recent study, De Lafontaine *et al.* (2013), by assessing the genetic diversity of *F. sylvatica* population at both the rear and leading edges, validated the expectation of isolated and drifting populations at the rear edge. Geber & Griffen (2003) in their synthesis of heritability values of plant traits (morphological, phenological and physiological traits generally studied in common garden experiments) found a mean heritability of 0.36 ± 0.297 (number of traits $\times$ populations = 1214), with a strong prevalence of low heritabilities (< 0.15) that accounted for nearly a half of the values. Thus, most our estimates are comparable to those found in other studies, and rather fall in the higher part of the range. It may be important to mention that the vast majority of the estimates provided by Geber & Griffen (2003) concerns short-lived species and long-lived species such as trees being under represented in heritability studies by this time. The first recently available Qst estimates for ecophysiological traits suggest that considerable adaptive genetic variation is harboured within tree populations (Ramirez-Valiente *et al.*, 2010; Alberto *et al.*, 2013). With this *in situ* study, by validating these findings on an adult population for traits directly measured *in situ*, we provide important complementary insights to the question of natural tree populations to respond to selection.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Heritability estimates using the Ritland's regression method as a function of the pairwise cut-off distances (in metres) for the following traits: leaf area (LA), leaf mass (LM), leaf mass per area (LMA), leaf nitrogen content (Nmass), leaf carbon isotopic discrimination (D13C) and leaf unfolding phenology (PSS). The Wang estimator was used to obtain pairwise relatedness estimates from molecular marker data. The actual variance of relatedness Vr_{ij} was used for computations (see section 'Estimation of relatedness coefficients' in Material and methods). Point estimates are represented by the blue straight line and the 95% bootstraped confidence intervals by the dotted grey lines (obtained using bootstraps, see section 'Test of statistical significance' in Materials and methods).

Table S1 Characteristics of the microsatellite loci and population genetic parameters of *Fagus sylvatica*. *n* is the number of observed alleles per locus, H_o is the observed heterozygosity, H_e is the expected heterozygosity.

Table S2 Heritability coefficients for the studied functional trait using the Ritland's linear regression method. The models presented here were estimated using the corrected variance of relatedness [**Var (r_{ij})**, see Materials and methods]. We presented the models performed using a 15, 30 and a 100 m cutoff distance. In bold are the point estimate of traits' heritability and in brakets are the lower and upper bounds of the 95% confidence interval. Traits' name corresponding to the abbreviations given here are listed in Table 1.

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