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Adaptive responses for seed and leaf phenology in natural populations of sessile oak along an altitudinal gradient

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Abstract

We assessed the adaptive potential of seed and leaf phenology in 10 natural populations of sessile oak (*Quercus petraea*) sampled along two altitudinal transects using common garden experiments. Population differentiation for both phenological traits was observed with high-altitude populations germinating and flushing later than low altitude ones. However, high genetic variation and heritability values were also maintained within populations, despite slightly decreasing for dates of leaf unfolding with increasing altitude. We suggest that biotic and abiotic fluctuating selection pressures within populations and high gene flow are the main mechanisms maintaining high genetic variation for these fitness related traits. Moreover, changes in selection intensity and/or selection pressures along the altitudinal gradient can explain the reduction in genetic variation observed for leaf phenology. We anticipate that the maintenance of high genetic variation will be a valuable resource for future adaptation of sessile oak populations undergoing an upslope shift caused by climate change.

Introduction

During the last decade, adaptive responses of animal and plant species to climate change have been increasingly investigated (reviews by Parmesan, 2006; Bradshaw & Holzapfel, 2008). One major concern in this context arose about the ability of trees to respond to climate change (Saxe *et al.*, 2001; Lindner *et al.*, 2010). Although palinological and phylogeographical studies on temperate tree clearly showed that they experienced several environmental changes in the past (Brewer *et al.*, 2002; Petit *et al.*, 2003; Magri *et al.*, 2006), their responses to future changes remain speculative (Kremer, 2007; Aitken *et al.*, 2008). The succession of cold and warm periods during the quaternary era came along with large changes in the geographic range of tree species, and subsequent adaptation to these changes is suggested by extant population differentiation (Kremer *et al.*, 2010). But nowadays, climate change caused by human activities is occurring at an unprecedented rapid rate (IPCC, 2007). According to Aitken *et al.* (2008), expected responses of tree populations to ongoing environmental changes have been sketched in three scenarios: migration, adaptation or extirpation. In mountainous landscapes, an upslope shift of plant species has already been observed (Lenoir *et al.*, 2008), although the fragmentation of habitats may limit range shifts (Davis & Shaw, 2001). Extirpation because of climate change has been predicted by niche modelling (Thomas *et al.*, 2004), but not yet observed. Local adaptation as a response to ongoing climate change is less tractable, although micro-evolutionary studies suggested rapid adaptations (Daubree & Kremer, 1993; Skroppa & Kohlmann, 1997). Here, we explore the capacity of oak adaptation to climatic changes by a retrospective analysis of population differentiation and diversity for seed and bud phenological traits along steep temperature gradients mimicking future environmental change.

Along with phenotypic plasticity of genotypes, which permits a fast but limited response to environmental

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changes, the level of genetic diversity of natural populations will be critical for future adaptation of tree species (Jump *et al.*, 2009). Local adaptation witnessed by genetic differentiation among populations feeds on genetic diversity but also on external gene flow (Aitken *et al.*, 2008). Indeed, high latitude and altitude populations will benefit from gene flow from central populations, which will introduce alleles preadapted to warmer climates (Davis & Shaw, 2001). It is therefore of crucial importance to estimate the extent of within- and between-population genetic variation in an ecological context, e.g. when populations are linked by gene flow. The distribution of genetic variability of tree populations for adaptive traits can be assessed in common garden experiments. These experiments were initially used by foresters as provenance tests, to monitor genetic variation for traits of economical and ecological relevance within a set of natural populations originating from different parts of the natural range (see for example Ducousso *et al.*, 1996). These studies were generally conducted to establish seed transfer zones and manage the genetic resources of tree species with a forestry perspective (Morgenstern, 1996), but they also provided estimates of the level of genetic variation in natural populations.

Among the most important adaptive traits, phenology is probably one of the most affected by global change (Bertin, 2008). Indeed, modifications of the phenology of flowering, bud burst or bud set in relation to global warming have been observed for many tree species (Menzel *et al.*, 2006; Nordli *et al.*, 2008) and predicted by mechanistic models (Bennie *et al.*, 2009). Later bud set and earlier bud burst increases the length of the growing season and the amount of net assimilation and the competitive ability of trees (Bennie *et al.*, 2009; Vitasse *et al.*, 2009a), whereas earlier bud set and later bud burst will improve cold resistance (Howe *et al.*, 2003). Variation in the timing of bud set and bud burst will also result in different synchrony with insect herbivores and fungal pathogens (Visser & Holleman, 2001; Van Asch *et al.*, 2007; Ghelardini & Santini, 2009). Modelling approaches have shown that temperature was the main driver of the timing of bud burst for most temperate tree species, along with photoperiod for few species (Chuine & Cour, 1999). Overall, bud burst is thought to be strongly related to the fitness of trees. Local adaptation of populations for bud burst in relation to climatic conditions has been described in several tree species and clinal variations have been widely reported according to latitude or altitude (Wright, 1976; Worrall, 1983; Morgenstern, 1996; Vitasse *et al.*, 2009b). Generally, tree populations from colder climates tend to flush earlier than populations from warmer climates when grown in common gardens. However, opposite temperature trends were also reported for the same species depending on the environmental gradient. Thus, southern and high-altitude provenances of sessile oak (*Quercus petraea*) showed earlier bud burst compared with northern and low altitude provenances (Liepe, 1993; Deans & Harvey, 1995; Ducousso *et al.*, 1996). Hence, the clinal patterns of differentiation for bud burst phenology in tree species requires further investigations to elucidate the sources of the differences among species and among populations within species. The timing of germination is also an important ecological trait intensively studied in the frame of plant adaptation to their environment (Donohue *et al.*, 2005; Finch-Savage & Leubner-Metzger, 2006). Young seedlings are indeed exposed to the same selection pressures as flushing buds, but they are critically more vulnerable to injuries than adult trees. The timing of seed germination has been the focus of intensive study, mostly in annual or biannual plant species that displayed a high variance both within and among seasons despite the strong selection expected on this trait (Donohue *et al.*, 2005; Simons & Johnston, 2006; Evans *et al.*, 2007; Venable, 2007; Simons, 2009). This diversification was considered as the archetypal illustration of the bet-hedging theory that hypothesises that a trait submitted to unpredictable environmental variation is selected for its variance rather than for its value (Cohen, 1966). Bet-hedging strategy thus corresponds to the production of a high phenotypic variance by a given genotype to maximize its long-term fitness under fluctuating selection (Simons, 2009). In trees, the pattern of variation for germination timing along latitude has been described for only a few species. Earlier germination of northern populations was observed for *Alnus sinuata* (Benowicz *et al.*, 2000) *Betula papyrifera* (Benowicz *et al.*, 2001) and *Tsuga heterophylla* (Campbell & Ritland, 1982), whereas the opposite cline was found for *Abies amabilis* (Davidson *et al.*, 1996). For *Quercus petraea*, the genetic differentiation of the timing of germination along altitude or latitude is yet totally unknown.

The main objective of this study was to estimate the level of genetic variability of seed and bud phenology in sessile oak populations located in the Pyrenees along two altitudinal transects. Sessile oak (*Quercus petraea* Matt. Liebl.) is a widespread European white oak species extending from Great Britain to the Iberian peninsula and from eastern Poland to the Atlantic Ocean. We used common garden experiments to quantify the genetic variability within and among populations sampled within natural populations connected by gene flow. Our objective was to estimate the impact of past divergent selection induced by the altitudinal gradient, and the capacity of populations to maintain genetic diversity. In other words, can local diversity be maintained despite local adaptation? Our second objective was to compare the distribution of genetic variation between two phenological traits (timing of seed germination and bud burst) that follow dormancy release and may therefore be the consequence of similar physiological pathways (Rohde *et al.*, 2000; Derory *et al.*, 2006; Rohde & Bhalerao, 2007; Ruttink *et al.*, 2007). Our investigations may lead to practical

applications for future research regarding the discovery of candidate genes for these traits.

Materials and methods

Sampling populations

Sessile oak (*Quercus petraea*) belongs to the complex of the white European oaks and is rather mesoxerophyle, preferentially located in south aspects and on acid soils. In the Pyrenees Mountains, sessile oak ranges from almost pure populations in the plain to more scattered stands at higher altitudes, reaching up to 1800 m. Natural populations of sessile oak located in two valleys (Ossau and Luz) in the Pyrenees Mountains (south-west of France) were sampled for this study. In each valley, five populations were sampled along an altitudinal gradient ranging from 131 to 1630 m (see Table 1 and Alberto *et al.*, 2010 for details). Earlier *in situ* investigations were conducted on phenological traits (Vitasse *et al.*, 2009a,c), and genetic diversity was monitored (Alberto *et al.*, 2010) on the same set of populations. In each population, air temperature was monitored every hour from year 2005 to 2009 using data loggers (HOBO Pro RH/Temp; Onset Computer, Bourne, MA, USA) as described in Vitasse *et al.* (2009a).

Harvesting of acorns

Acorns were harvested in September 2006 on 152 adult trees within the 10 natural populations, resulting in a total collection of 8833 acorns (Table 1). Depending on the level of fruiting within each population, open-pollinated progenies were collected from 7 to 33 trees per population. Monitoring of leaf unfolding in the spring was conducted in 2005, 2006 and 2007 on the mother trees of the collected progenies (Vitasse *et al.*, 2009c). The number of acorns varied greatly by population and mother tree because of the heterogeneity of the seed crop. Acorns were weighed for each open-pollinated progeny. The average weight of acorns was homogeneous between families of the same population but decreased among populations with respect to altitude (Table 1). Acorns were stored in a cold room at + 4 °C during the following winter to ensure chilling requirements prior to the germination.

Sowing and plantations establishment

Acorns from the 152 open-pollinated families were sown in a greenhouse at the INRA Pierroton station (PIE; 44°44′N, 00°46′W) in March 2007 within a complete block design (152 entries in five blocks), but the number of acorns per entry per block varied because of the uneven seed crop (on average 12 acorns per entry per block). Sowing was carried out on individual clods of loam homogeneously covered by levelled sand in small containers. The overall germination rate was 52.4% but differed among provenances from 73.7% (L3) to 28.8% and 27% (O16 and O4, respectively). In winter 2007, the containers with germinated acorns were transferred in a common garden experiment located in the nursery of the INRA research station at Pierroton (PIE; 44°44′N, 00°46′W). The experimental plantation comprised complete blocks with random spatial allocation of entries within blocks. Seedlings were planted in rows with 1 × 0.5 m spacing. During autumn 2008, seedlings were uprooted and transplanted in a field site located on the

Table 1 Description of the sample size of provenances in each common garden.

Prov *	Altitude *in situ* (m)	Altitudinal level (m)	Altitudinal group†	Mean acorn weight (g)	PIE (sowing and plantation)				TOUL	
					N_f ‡	N_{acorns}	N_{GERM}§	N_{LU} ‡	N_f‡	N_{LU}¶
L1	131	100	LA	2.81	11	324	150	163	11	110
L3	387	400	LA	2.80	18	471	336	344	18	228
L8	803	800	LA	1.73	16	1094	345	479	16	323
L12	1235	1200	HA	2.20	14	468	248	282	14	191
L16	1630	1600	HA	0.97	14	829	291	445	14	349
O1	259	100	LA	2.51	24	1605	456	592	23	428
O4	422	400	LA	3.38	7	126	34	33	7	29
O8	841	800	LA	2.41	8	721	188	348	7	262
O12	1194	1200	HA	2.05	33	2611	1090	1744	33	1358
O16	1614	1600	HA	0.88	7	584	131	166	7	103
Total				2.17	152	8833	3269	4596	150	3381

*Prov: Provenance codes. Codes beginning with letter L and O indicate provenances from Luz valley and Ossau valley, respectively.
†LA: provenances coming from altitudes below 1000 m, HA: provenances coming from altitudes above 1000 m.
‡N_f: number of open-pollinated families per provenance.
§N_{GERM}: number of individuals measured for germination timing per provenance in Pierroton (PIE).
¶N_{LU}: number of individuals measured for leaf-unfolding timing per provenance in Pierroton (PIE) and in Toulenne (TOUL).

fruit tree research field of INRA at Toulenne (TOUL; 44°34′N, 00°16′W), very close to the nursery where the plants were raised. The plantation comprised 3448 plants representing 150 families (Table 1). Five randomized complete blocks were set up in a 3.5×1.5 m density plantation. Mortality was 67 plants in 2009 and 39 plants in 2010. Throughout the paper, the term population refers to the trees in the natural stands on which acorns were harvested, and the term provenance refers to the offspring of these mother trees, which were sown and planted in the common garden experiments. Individual identity of seedlings (provenance, family within provenance and individual within family) was maintained during the whole experiment.

Phenological measurements

Seed germination was recorded in the greenhouse at PIE during spring 2007 on four blocks corresponding to a subset of 6475 acorns. The date of shoot emergence out of the sand surface was recorded and considered as an indirect assessment of the time of germination. Previous observations have indeed shown that despite a short lag, there was a high correlation between the time of root and shoot emergence (F. Alberto, personal observations). Date of emergence of each acorn was scored as the number of days since 1 January (DOY, day of the year) when the emergence of the shoot was observed (GERM). Monitoring was conducted twice per week on the whole set of germinated acorns.

The development stage of the apical bud from fully dormant bud to internodes elongation was monitored on each seedling according to Derory *et al.* (2006). Scoring was carried out on each seedling twice a week during spring 2008 at PIE and during springs 2009 and 2010 at TOUL (TOUL1 and TOUL2, respectively). Leaf-unfolding stage, corresponding to at least one leaf completely unfolded out of the bud, was here considered as the target trait, and the day when leaf unfolding was observed was recorded (LU). Linear interpolation was used to estimate LU when leaf unfolding occurred earlier or later then the day of observation. The number of individuals measured for GERM and LU are presented in Table 1.

For all mother trees sampled within the ten populations, leaf unfolding has also been previously monitored *in situ* on the mother trees during springs 2005, 2006 and 2007 following the same procedure (Vitasse *et al.*, 2009c). Yearly *in situ* observations of LU were transformed as standardized values to account for population differences recorded *in situ*.

Statistical analyses

Quantitative genetic analyses

GERM and LU were analysed to partition the phenotypic variance into its genetic and environmental components. Several mixed models were fitted using a restricted maximum likelihood method with the ASReml software (Gilmour *et al.*, 2002).

Genetic parameters were first estimated in each site and year separately. The following mixed model was used for each phenological variable, GERM and LU at PIE, TOUL1 and TOUL2:

$$Y_{ijk} = \mu + b_k + P_i + F_{j(i)} + (Pb)_{ki} + (Fb)_{kj(i)} + \varepsilon_{ij}$$

where μ is the trait mean, b_k is the fixed block effect, P_i and $F_{j(i)}$ are random effects for provenance and family within provenance, respectively, $(Pb)_{ki}$ and $(Fb)_{kj(i)}$ are the interactions between block and random effects and ϵ_{ijk} is the residual effect. The significance of each effect was estimated by a likelihood ratio test (threshold at 0.05).

The model provided an overall estimate of the within population additive genetic variance (σ^2_{G}). While we were also interested in comparing the within-population additive genetic variance (σ^2_{G}) along the altitudinal gradient, the model was also run within altitudinal classes: either by bulking populations of the same altitude between the two valleys or by bulking populations of low (LA) and high (HA) altitudes (see Table 1 for details).

Genetic parameters and differentiation estimates

The phenotypic variance within provenances (σ^2_{Ph}) was estimated as: $\sigma^2_{\mathrm{Ph}} = \sigma^2_{\mathrm{F}} + \sigma^2_{(\mathrm{bf})} + \sigma^2_{\varepsilon}$. In both models, the additive genetic variance (σ^2_{G}) was estimated as: $\sigma^2_{\mathrm{G}} = 4\mathrm{x}\sigma^2_{\mathrm{F}}$ (σ^2_{F} is the variance of family within provenances) assuming that open pollinated offspring were half-sibs and no maternal effects.

We tested the significance of the differences between the genetic variances of the altitudinal classes using a *Z* test.

The genetic differentiation among populations was estimated by the Q_{ST} that expresses the amount of provenance variation (σ^2_{P}) related to the overall genetic variation (Spitze, 1993) such that:

$$Q_{\mathrm{ST}} = \sigma^2_{\mathrm{P}}/(\sigma^2_{\mathrm{P}} + 2 \times \sigma^2_{\mathrm{G}})$$

Narrow-sense heritability h^2 was estimated as:

$$h^2 = \sigma^2_{\mathrm{G}}/\sigma^2_{\mathrm{Ph}}$$

Finally, breeding values (e.g. additive genetic values) of each mother tree and provenance values were estimated for GERM and LU using best linear unbiased predictors in ASReml. The standard errors of genetic parameters were calculated with ASReml using a standard Taylor series approximation (Gilmour *et al.*, 2002).

To make a comparison with the estimation by mixed linear model, the heritability of LU was also estimated through the parent–offspring relationship. According to Lynch & Walsh (1998), in the case of half-sibs the heritability can be directly estimated from the regression coefficient b_{op} of the parent–offspring regression, where $h^2_{\mathrm{reg}} = 2 \times b_{op}$. Linear regressions were conducted between the standardized values of LU measured in common gardens (at PIE, TOUL1 and TOUL2) and the standardized

values of LU measured *in situ* in 2006 using the statistical package R (R Development Core Team, 2005). We performed the linear regressions and calculated heritability for the overall sample and for each altitudinal class.

Q_{ST} and F_{ST} comparison

To assess whether differentiation of phenological traits was caused by selection, we compared the distribution of phenotypic differentiation (Q_{ST}) with the distribution of genetic differentiation (F_{ST}) assessed with molecular markers assumed to be neutral (Waldmann *et al.*, 2005; Goudet & Buchi, 2006; Goudet & Martin, 2007). The F_{ST} distribution was constructed using a dataset of 14 neutral nuclear microsatellites previously genotyped on the same populations (Alberto *et al.*, 2010). To simulate the neutral expectation, we resampled randomly 10^3 times with replacement between loci to estimate the sampling variance of F_{ST}. Each F_{ST} replicate value was multiplied by a random number drawn from the Lewontin & Krakauer (1973) distribution, which accounts for deviations from the neutral model because of demography (Whitlock, 2008; Whitlock & Guillaume, 2009). The Q_{ST} distributions were constructed by performing a parametric bootstrap resampling procedure 10^3 times (O'Hara & Merila, 2005) allowing estimation of the distribution of each variance component (σ^2_G, σ^2_P) using the Satterthwaite's approximation (Satterthwaite, 1946). Finally, the two resulting distributions were compared using a Wilcoxon test.

Correlations between traits and environmental variables

Correlations between environmental variables and traits were tested by linear regressions conducted with the *lm* function using R software (R Development Core Team, 2005). The significance of each linear regression was tested using Fisher's test. We considered the provenance values and the family breeding values of GERM and LU estimated in the mixed model instead of provenance and family means. First, the potential effect of the amount of resources contained in acorns (acorn weight) on the phenology of germination was tested. Linear regressions between GERM and acorn weight were performed using the average acorn weight at the provenance level and the standardized values of acorn weight at the family level. Second, to link the among-provenances differentiation with environmental variables, we performed linear regressions between provenance values and either altitude or spring temperatures in 2006 of the provenances' sites of origin.

Finally, we calculated the genetic correlation between GERM and LU by linear regression between family and provenance values of both traits and the phenotypic correlation by computing at the single tree level.

Late spring frost occurrence

To assess whether late spring frosts may be a selective driver of phenological traits, we used the temperature data recorded *in situ* during years 2005 to 2009 to calculate their occurrence in natural populations. We considered that negative minimum daily temperatures corresponded to one frost event, and we counted the number of frost events occurring at most 15 days before the first tree flushed in a given population. Thus, we only retained late spring frosts that could represent a selective pressure for both phenological traits, knowing that frost damages only flushing tissues and not dormant ones. Finally, we compared the occurrence of frost events among natural populations along the altitudinal gradient.

Results

Provenance differentiation

For both models, main effects were all significant, whereas interactions effects were not (Table 2). Provenances differed greatly as shown by the high Q_{ST} values estimated for GERM (0.29) and LU (0.23 in PIE, 0.21 in TOUL1 and 0.16 in TOUL2). The comparison of distributions between Q_{ST} values obtained for both phenological traits and the F_{ST} value calculated from the neutral microsatellites (F_{ST} = 0.02; Alberto *et al.*, 2010) showed that differentiation among populations for all traits was more important than expected under neutrality ($P < 0.001$ for all comparisons, data not shown), indicating strong diversifying selection. Both phenological traits, GERM and LU, followed a consistent clinal trend of variation according to altitude. Provenances from high altitudes germinated and flushed later than populations from low altitudes (Fig. 1).

Table 2 Genetic parameters estimated for phenological traits in the common garden experiments.

Trait	GERM	LU (PIE)	LU (TOUL1)	LU (TOUL2)
Mean DOY	119.5	107.8	108.9	118.5
Q_{ST}	0.28 (0.11)	0.23 (0.09)	0.21 (0.09)	0.16 (0.07)
h^2	0.51 (0.08)	0.87 (0.11)	1.07 (0.13)	0.98 (0.12)
σ^2_G	117.7 (20.2)	31.7 (4.9)	30.4 (4.9)	23.2 (3.7)
σ^2_{Ph}	232.2 (7.0)	36.6 (1.3)	28.5 (1.3)	23.7 (1.0)
σ^2_P	90.2	18.6	15.7	8.8
σ^2_F	29.4	7.9	7.6	5.8
σ^2_{Fb}	–	1.7	2.0	1.3
σ^2_{Pb}	–	0.3	–	–
σ^2_ϵ	202.8	26.9	18.9	16.6

For germination (GERM) and leaf unfolding (LU) dates at Pierroton (PIE) and Toulenne (TOUL1 and TOUL2 for year 1 and 2, respectively), the mean day of the year (DOY) over all provenances is given.

For each analysis, the among-provenances differentiation ($Q_{ST} = \sigma^2_P/(\sigma^2_P + 2 \times \sigma^2_G)$) and the heritability ($h^2 = \sigma^2_G/\sigma^2{}_{Ph}$) were calculated from the provenance variance σ^2_P, the genetic variance σ^2_G and the within-provenance phenotypic variance σ^2_{Ph}. Standard errors are given between parentheses. Variances associated with the mixed model (σ^2_F, σ^2_{Fb}, σ^2_{Pb} and σ^2_ϵ) are described into the text.

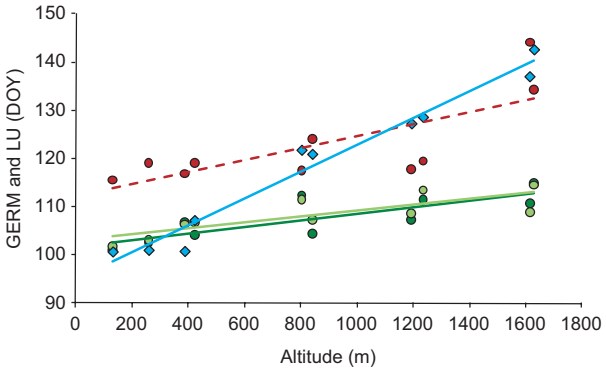

Fig. 1 Altitudinal trends of variation for germination and leaf unfolding. Each point represents the provenance mean of germination (GERM) or leaf unfolding (LU) dates according to the altitude of the provenance's site of origin. GERM is in dark red circles, LU is in dark green circles at Pierroton (PIE), in light green circles at Toulenne the first year (TOUL1), in yellow green circles at Toulenne the second year (TOUL2), and LU *in situ* measured in 2007 is in light blue diamond-shaped points. The regression lines are represented in the corresponding colours with dashed lines significant at the 0.05 level and solid lines at the 0.01 level.

However, altitudinal clines exhibited slight differences between GERM and LU. Altitudinal variation for GERM was rather discontinuous, whereas provenances coming from up to 1200 m germinated between DOY 116 and 124, and provenances from high altitude (1600 m) germinated much later (between DOY 134 and 144 for L16 and O16, respectively). The timing of leaf unfolding displayed a continuous clinal pattern of variation that was stable among sites and years. In PIE and TOUL1, the pattern was almost identical with the earliest provenance flushing on average at DOY 101 and the latest at DOY 115 (respectively, provenances L1 and L16). Delays of LU by altitude amounted on average to 0.61 and 0.69 days per 100 m increase in altitude at TOUL1 and PIE, respectively. Flushing period was later in TOUL2, where the earliest flushing occurred at DOY 113 and the latest at DOY 123. The clinal pattern was however consistent with PIE and TOUL1, despite the slightly lower rate of change (0.47 days per 100 m).

Within-population genetic variation

Family effects were highly significant in both models used to estimate components of the phenotypic variance. According to model 1, we found high estimates of heritability for both phenological traits (Table 2). The heritability of GERM amounted to 0.52. This trait displayed high phenotypic and additive genetic variances within provenances (Table 2). Although the additive genetic variances of LU were lower during the 3 years of measurement, LU exhibited higher heritability values (h^2 = 0.87 in PIE and h^2 = 1.07 and 0.98 at TOUL1 and TOUL 2, respectively).

Standardized values of LU measured on the offspring in PIE were highly correlated with those measured on mother trees *in situ* in 2006 (R^2 = 0.23, d.f. = 149, $P = 3.95 \times 10^{-10}$; Fig. 2). The correlation was also highly significant with LU measured at TOUL1 and TOUL2 (R^2 = 0.28, d.f. = 146, $P = 5.89 \times 10^{-12}$ and R^2 = 0.22, d.f. = 145, $P = 2.57 \times 10^{-9}$, respectively). Linear regressions performed with *in situ* measurements in 2005 and 2007 were also highly significant, but correlation coefficients were lower (data not shown). The heritability values estimated by parent–offspring regressions (h^2_{reg} = 0.94 at PIE, Fig. 2; and h^2_{reg} = 1.01 and 0.91 at TOUL1 and TOUL2, respectively) were of the same order of magnitude as the estimated values obtained by the intraclass correlation coefficients within the open-pollinated progenies. However, when calculated by altitudinal classes, we found slight discrepancies between both estimates (Table 3). These discrepancies may be either because of the assumption of half-sib relationships within the open-pollinated progenies or because of the varying environmental variances occurring between the *in situ* forest environment and the common garden experiment. Nevertheless, the estimates of heritability by the two methods were always higher than 0.50 (except for the 1200 m populations with h^2 = 0.44 at TOUL2).

When variance components were separately estimated within altitudinal classes, there was a general trend of decrease in genetic variation of LU with increasing altitude (Table 3). Indeed, the genetic variances of LU were dramatically lower for the high-altitude group compared to the low-altitude group for the 3 years (*Z* test, $P < 0.05$ at PIE and TOUL1 and $P < 0.01$ at TOUL2, Table 3). Between altitudinal levels, genetic variances decreased from 70.9 at 400 m to 19.5 at 1200 m of altitude at PIE. Differences were significant when com-

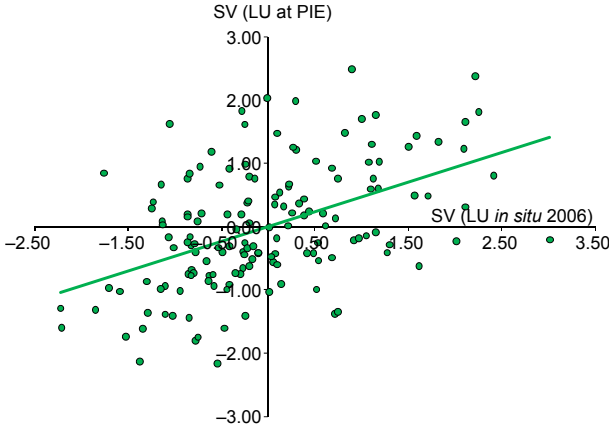

Fig. 2 Parent–offspring relationship for leaf unfolding. Linear regression between standardized values (SV) of leaf-unfolding dates measured on offspring at Pierroton (LU at PIE) and leaf-unfolding dates measured on mother trees in natural populations in 2006 (LU *in situ* 2006).

Table 3 Main genetic parameters estimated by grouping provenances according to altitude.

	Altitudinal levels					Altitudinal groups	
	100 m	400 m	800 m	1200 m	1600 m	LA	HA
GERM							
Mean DOY	115.4	115.8	118.2	117.4	137.2	116.5	122.1
h^2	0.87 (0.22)	0.3 (0.18)	0.47 (0.18)	0.26 (0.08)	0.67 (0.25)	0.63 (0.13)	0.44 (0.1)
σ^2_G	184.7 (59)	46.9 (29.4)	99.2 (42.2)	52.9 (17.1)	292.9 (130.7)	125.8 (29)	114.8 (28.4)
LU (PIE)							
Mean DOY	102.4	106.0	109.4	107.8	113.1	106.0	109.0
h^2	0.87 (0.23)	1.37 (0.37)	0.75 (0.24)	0.61 (0.14)	0.77 (0.28)	1.00 (0.16)	0.66 (0.13)
h^2_{reg} †	1.01***	0.92**	1.51***	0.59*	1.23**	1.05***	0.78**
σ^2_G	37.7 (12.4)	70.9 (28.2)	28.9 (11.0)	19.5 (5.2)	21.9 (9.5)	42.8 (9.0)	20.5 (4.6)
LU (TOUL1)							
Mean DOY	103.3	106.8	110.6	109.4	112.9	107.0	110.2
h^2	1.05 (0.27)	1.58 (0.40)	0.66 (0.26)	0.65 (0.16)	1.10 (0.40)	1.15 (0.19)	0.76 (0.16)
h^2_{reg} †	1.14***	1.35***	0.73 ns	0.83**	1.05**	1.10***	0.90***
σ^2_G	42.4 (14.1)	67.0 (26.9)	19.3 (8.9)	12.8 (3.7)	27.8 (13.4)	42.9 (9.5)	15.9 (4.0)
LU (TOUL2)							
Mean DOY	114.3	117.3	119.5	118.8	122.3	117.1	119.6
h^2	1.19 (0.28)	1.45 (0.38)	0.96 (0.29)	0.44 (0.13)	0.87 (0.36)	1.23 (0.19)	0.57 (0.13)
h^2_{reg} †	1.05***	1.01**	0.98*	0.67*	1.06*	1.00***	0.79**
σ^2_G	40.2 (12.9)	41.9 (16.7)	22.3 (8.8)	8.0 (2.5)	18.8 (9.4)	36.0 (7.6)	19.0 (0.9)

†The significance of h^2_{reg} corresponds to the p-value of the linear regressions conducted between the standardized values of LU measured at PIE and LU measured *in situ* in 2006. Symbols meanings: ns $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Standard errors are given between parentheses. See into the text for abbreviations.

paring the provenance from 1200 m with provenances of 100 and 400 m at TOUL1 and TOUL2 ($P < 0.05$) but not at PIE (Table 3). The combined provenance and genetic variation is illustrated by the distribution of the mean values of leaf unfolding within each population (Fig. 3). The overall picture was the maintenance of a large within-population genetic variation associated with a clinal population differentiation along the altitudinal gradient. The latest flushing tree from the lowest altitude unfolds its leaves at about the same period as the earliest flushing tree from the highest altitude.

While timing of germination also exhibited a large genetic variation (Table 2), the genetic variance σ^2_G of GERM did not show any altitudinal trend (Table 3), as genetic variances were not significantly different between altitudinal groups. The greatest σ^2_G values were found for provenances from the lowest and highest altitudes (184.7 and 292.9 for 100 and 1600 m, respectively), whereas the lowest value was found at 400 m (46.9).

Correlations between traits and environmental variables

We found no genetic correlation between family and provenance values of GERM and LU ($R^2 = 0.00$, d.f. = 150, $P = 0.40$ and $R^2 = 0.19$, d.f. = 8, $P = 0.20$, respectively). Heavier acorns germinated significantly faster ($R^2 = 0.59$, d.f. = 8, $P = 0.0093$) at the provenance level. This regression was mainly driven by the late germination of provenances from 1600 m for which acorns weighed much less than those of others provenances (Table 1). The regression was also significant between acorn weight and LU at PIE ($R^2 = 0.62$, d.f. = 8, $P = 0.0069$) and TOUL2 ($R^2 = 0.44$, d.f. = 8, $P = 0.0365$) but not at TOUL1 ($R^2 = 0.38$, d.f. = 8, $P = 0.0535$). The slopes of the linear regressions decreased from −8.92 for GERM to −4.14, −3.01 and −2.37 for LU at PIE, TOUL1 and TOUL2, respectively. However, acorn weight was not correlated with the variation observed among families within provenances for any trait ($P > 0.05$, data not shown).

The regression between provenance values and environmental variables of the sites of origin were highly significant (Table 4). The provenance values of GERM were best correlated to spring temperatures, whereas LU exhibited stronger correlations with altitude.

Late spring frost occurrence

A total of 31 frost events occurring before the flushing period were recorded during year 2005–2009 along the altitudinal gradient. These late spring frosts occurred between DOY 79 in 2007 in L1 population and DOY 137 in 2005 in population L16, but none was recorded in populations O1 and L12. When grouping populations by altitudinal levels, the occurrence of frost events increased

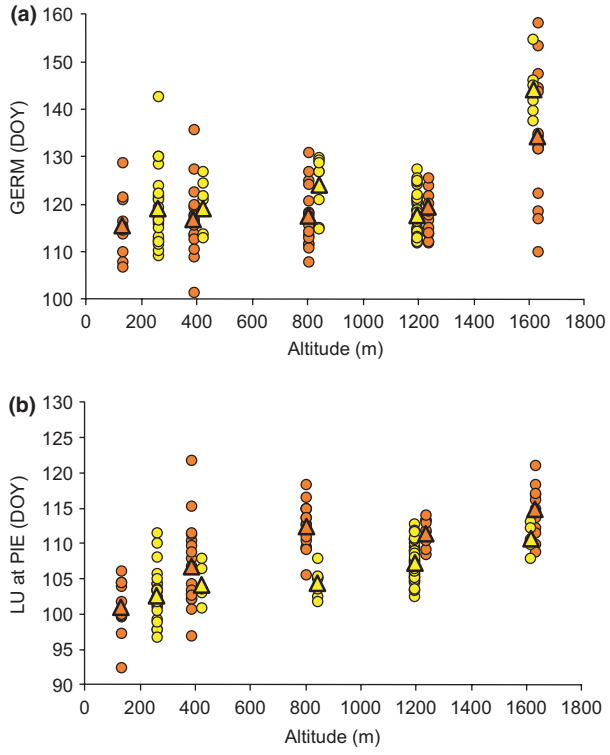

Fig. 3 Variability of (a) germination and (b) leaf unfolding along the altitudinal gradient. The triangle-shaped points represent the mean of the provenances and the circles represent the mean values of the families of (a) GERM and (b) LU measured at Pierroton (PIE), in orange for Luz valley and in yellow for Ossau valley. The horizontal axis corresponds to the altitude of provenances' sites and the vertical axis corresponds to the DOY of GERM and LU at PIE.

significantly along the altitudinal gradient (R^2 = 0.97, d.f. = 3, P = 0.0023; Fig. 4). There were, for example, only two frosts in populations from 100 and 400 m compared to 9 and 12 frosts recorded in population from 1200 and 1600 m, respectively.

Discussion

Seed and leaf phenology displayed significant levels of differentiation among provenances in common garden experiments. Differentiation of both traits followed a clear and strong altitudinal trend, with provenances from high-altitude displaying later phenology than provenances from low altitude. In addition, differentiation coexisted with very large within-population genetic diversity. These results suggest peculiar interplays between gene flow and natural selection ensuring local extant adaptation. We suggest that these mechanisms may also allow populations to cope with future environmental changes.

Heritability and maternal effects

Seed and bud phenology exhibited very large heritability values in our experiments. Strong genetic control of bud phenology in forest tree species was reported in earlier studies (review in Howe *et al.*, 2003). In our study, high heritability values may have a three-fold source: a low environmental variance, large genetic variances and maternal effects.

The latter interpretation deserves special attention as maternal effects may be important in trees for traits assessed on juvenile seedlings and upwardly bias genetic variances. Maternal effects may have an environmental or genetic component. Environmental maternal effects would be induced by the common ecological conditions where the mother tree was growing *in situ* and under which seeds matured. These common environmental effects may further generate after effects on the seedlings raised in the nursery and field tests. However, if maternal environmental effects are not transmitted over generations, then they will not upwardly bias genetic variances estimated by parent–offspring regression (Rossiter, 1996; Kruuk & Hadfield, 2007). In our case, transgenerational maternal environmental effects would mean, for example, that locally cold temperatures contributing to late flushing of a maternal tree standing in the forest would also contribute to late flushing of all half-sib seedlings raised in the nursery or in the common garden. To date, we have no evidence of such transgenerational effects in oaks or broadleaved trees; but after effects have been reported in conifers for the timing of bud set in Norway spruce (Skroppa & Kohlmann, 1997). Assuming that transgenerational environmental maternal effects do

Table 4 Linear regressions performed between provenance values (PV) of germination and leaf unfolding and environmental factors of provenances' sites of origin.

	Altitude*				T_{spring}†			
	Slope	R^2	d.f.	P	Slope	R^2	d.f.	P
PV GERM	0.013	0.49	8	0.0139	−3.086	0.63	8	0.0058
PV LU (PIE)	0.006	0.67	8	0.0034	−1.365	0.61	8	0.0079
PV LU (TOUL1)	0.006	0.62	8	0.0068	−1.152	0.51	8	0.0197
PV LU (TOUL2)	0.004	0.64	8	0.0054	−0.892	0.56	8	0.0125

*Altitude: altitude (m) of the provenance's sites of origin; †T_{spring}: mean temperatures (°C) from 1st March to 31st May 2006. See into the text for abbreviations.

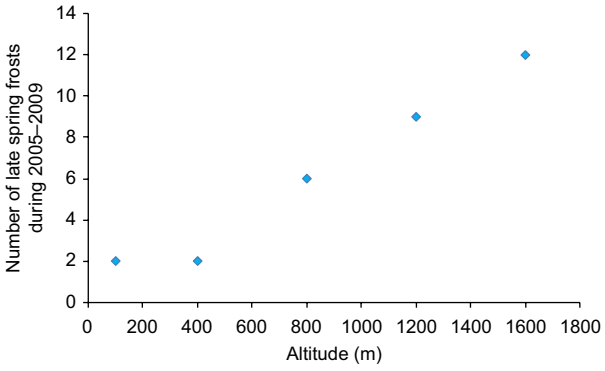

Fig. 4 Occurrence of frost events in natural populations during springs 2005–2009 along the altitudinal gradient. Populations were grouped by altitudinal levels. Frost events were recorded as negative minimal daily temperatures occurring at most 15 days before the first flushing tree in each population.

exist, then the bias on the estimated additive variances (σ^2_G) should amount to $2V_{EM}$ when σ^2_G is estimated by parent–offspring covariances and $4V_{EM}$ when σ^2_G is estimated by half-sib covariances. The same differences in bias would exist for genetic maternal effects ($2V_{GM}$ and $4V_{GM}$). Because heritability estimates with both methods were quite similar in our case, we conclude that maternal effects only moderately affected estimates of genetic variances. These conclusions are also supported by earlier reported estimates of heritability of bud burst in the closely related species *Quercus robur*. Heritability estimate of 0.87 was found at age 4 in Lithuanian populations of *Q. robur* (Baliuckas & Pliura, 2003), using parent–offspring correlations where parents and offspring were raised under different environmental conditions. Finally, in a single full-sib family of *Q. robur*, Scotti-Saintagne *et al.* (2004) found moderate-to-high heritability values (0.15–0.52).

Clinal variation and local adaptation

Adaptive variation in leaf unfolding in *Quercus petraea* followed an altitudinal cline that was consistent across the two experimental plantations and was also observed within each valley. Our results contradict earlier findings that showed an opposite altitudinal cline of bud burst phenology in the same species (Deans & Harvey, 1995; Ducousso *et al.*, 1996). However, in these two studies, all provenances originated from below 500 m a.s.l., where temperature inversions often occur. Because of the large sampling over the natural range of the species, altitudinal effects were also confounded with longitudinal and latitudinal effects. Indeed, populations from southern latitudes flush earlier (Ducousso *et al.*, 1996), and mountainous areas are mainly located under southern latitudes in Europe. In our case, sampling of populations within the same valleys allows to disentangle altitudinal from latitudinal trends. We confirmed the results of Vitasse *et al.* (2009b) who assessed altitudinal genetic differentiation of six woody species, of which *Q. petraea* coming from the same Pyrenean valleys. As already suggested by Vitasse *et al.* (2009b), the late flushing of high-altitude provenances could result from an adaptation to avoid leaf damages caused by late spring frosts, which occurs more frequently at high altitudes. Moreover, adaptive variation between populations of *Q. petraea* was congruent along altitudinal and latitudinal gradients with populations from colder climates displaying later flushing than populations from warmer climates (Liepe, 1993; Deans & Harvey, 1995; Ducousso *et al.*, 1996). Parallel clines for altitude and latitude suggest that the avoidance of late spring frosts either at higher altitudes or at latitudes may be the main selecting factor shaping population differentiation. This is a likely interpretation of our results, as the occurrence of late frosts increases with altitude (Fig. 4). Alternative hypothesis to natural selection were recently proposed to account for high population differentiation for bud phenology in other tree species, and particularly in Norway spruce. Indeed, it was shown that after effects may modify bud set of *Picea abies* (Skroppa, 1994). Whether the response is because of maternal or epigenetic effects (Johnsen *et al.*, 2005), the consequences lead to rapid population differentiation within one single generation (Skroppa & Kohlmann, 1997). Epigenetic effects have been rarely described in broad-leaved trees and are poorly understood (Rohde & Junttila, 2008), and their contribution to population differentiation of leaf unfolding in *Q. petraea* would deserve future investigations.

Altitudinal variation was also observed for the date of germination. Later germination of high-altitude populations was also found by Campbell (1979) on Douglas fir seeds. However, in our study altitudinal variation was caused by the very late germination of seed coming from the high-altitude populations. As for leaf unfolding, altitudinal trends may be the result of natural selection favouring late germinating seeds to avoid late frost that would be fatal for the young seedlings. It would certainly account for the very late germination of populations from the highest altitudes. However, as for leaf unfolding population differentiation may be inflated or blurred by maternal effects. Maternal environment effects have been reported in the seed germination of *Arabidopsis thaliana*. Donohue *et al.* (2005) showed that temperature, photoperiod and light quality during seed maturation influenced significantly the germination timing. Along the altitudinal gradient, temperature and light quality vary considerably between populations, generating differences in seed maturation that may subsequently induce variation of the timing of germination. Effects of seed size on the timing of germination have also been reported in several plant species without a common trend emerging among species (Kalisz, 1989; Zammit & Zedler, 1990; Platenkamp & Shaw, 1993; Simons & Johnston,

2000). Maternal provision is known to usually result in better-nurtured seeds that possess an obvious advantage for seedling establishment (Rohde & Junttila, 2008), as shown by positive correlations between seed weight and seedling development (Oleksyn *et al.*, 1998; Vitasse *et al.*, 2009b). When seeds contain few resources, a late germination thus appears as an advantageous strategy to ensure favourable conditions during seedlings emergence. In conclusion, earlier records of the effects of environmental conditions prevailing during seed maturation on seedlings traits moderate our interpretations about genetic differentiation resulting from natural selection on seed and leaf phenology.

Maintenance of genetic diversity within population

In our study, the range of within-population variation extends the range of the among-population variation (Fig. 3). Interestingly, this large genetic variation was maintained while local adaptation occurred at the same time. This result can be explained by the balance between selection pressures acting on leaf unfolding. First, high-population variance can be caused by environmental heterogeneity that can occur through space (Campbell, 1979) but also over years. Fluctuating environmental changes may modify the selective values of traits and represent a powerful force for maintaining genetic diversity within natural populations (Jump *et al.*, 2009). Whereas late frosts during spring can critically damage leaves of early-flushing trees, the lengthening of the growing season can be advantageous if frost is avoided (Vitasse *et al.*, 2009c). Second, different selection pressures acting on leaf phenology in opposing directions would also maintain large within-population variation. Besides the temperature-mediated effect on bud burst, the synchrony between bud flushing of oaks and the emergence of pest populations such as insect herbivores and/or fungal pathogens may also be a determinant factor of selection in natural populations. Desprez-Loustau *et al.* (2010) have recently studied the synchrony between *Erisyphe alphitoides*, the pathogen responsible of the oak-powdery mildew and oaks along the same altitudinal gradient in the Pyrenees and found that the pathogen was preferentially synchronized with late-flushing oaks at low altitudes. Hence, late frosts can damage early-flushing trees and select for late flushing, whereas pest populations can be synchronized with the late-flushing trees and thereby favouring early-flushing trees. However, the synchrony changed with altitude, and early-flushing oaks were more infected at high altitudes. This result can also explain the decreasing trend of genetic variation with altitude for bud phenology. If the balance between biotic and abiotic selective pressures is modified along the altitudinal gradient, changes of selection type can account for the reduction in genetic variation along the altitudinal gradient. Hence, the stronger intensity of directional selection generated by late spring frosts at high altitudes combined with a change in biotic interactions can contribute to the reduction in genetic variation with altitude. The preferential unidirectional gene flow from lower to higher altitudes (Alberto *et al.*, 2010) may however be also responsible for this trend. For germination timing, the level of within population variability is larger than for leaf unfolding but does not follow an altitudinal trend. Germination timing has been less investigated, although large variation was also observed within populations of Douglas fir (Campbell, 1979) and Pacific silver fir (Davidson *et al.*, 1996). We suspect that biotic and abiotic selection pressures shaped the distribution of genetic variation within populations just as for leaf unfolding. However, as germination timing is a highly critical stage for seedling survivorship, stronger diversification is expected for this trait. In this study, the diversification of germination timing is largely because of the high genetic variance although microenvironmental effects may also contribute to the variation existing among siblings of the same mother tree. In contrast to species where bet hedging was proposed (which are mainly selfing; Simons & Johnston, 2006), sessile oak is largely outcrossing resulting in large genetic variation between siblings. Our results therefore suggest that genetic effects may account for the large phenotypic variation observed for germination timing in this species. Finally, the lack of genetic correlation between germination and leaf unfolding suggest that different genes might be involved in the genetic control of both traits, and consequently that investigation aiming at the discovery of candidate genes should be conducted separately for each trait.

Microevolution and adaptation to climate change

We suspect that the population differentiation observed in our study result from diversifying selection prevailing along the altitudinal gradient. According to pollen records, the altitudinal colonization of *Q. petraea* in the Pyrenees lasted <2000 years and was achieved between 11 000 and 10 000 BP (Aubert, 2001; Belet, 2001). In the two studied valleys, phylogeographical interpretations using chloroplast markers indicated that common source populations located in the low-land plain colonized the altitudinal gradient (Alberto *et al.*, 2010). Hence, extant populations originated from the same gene pool and became locally adapted because of diversifying selection during the Holocene, while climate was progressively warming. Our results indicate that local adaptation along the altitudinal gradient occurred despite high gene flow connecting natural populations (Alberto *et al.*, 2010), while high levels of genetic variation were maintained. If microevolutionary changes were indeed generated by past natural changes, the extent of future adaptive responses to human-mediated climatic changes remains on open debate.

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