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Evolution, plasticity and evolving plasticity of phenology in the tree species *Alnus glutinosa*

H. DE KORT*, K. VANDER MIJNSBRUGGE†‡, K. VANDEPITTE*, J. MERGEAY†, O. OVASKAINEN§ & O. HONNAY*

*Plant Conservation and Population Biology, Biology Department, University of Leuven, Heverlee, Belgium
†Research Institute for Nature and Forest, Geraardsbergen, Belgium
‡Agency for Nature and Forest, Brussels, Belgium
§Department of Biosciences, University of Helsinki, Helsinki, Finland

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Abstract

Both traits and the plasticity of these traits are subject to evolutionary change and therefore affect the long-term persistence of populations and their role in local communities. We subjected clones from 12 different populations of *Alnus glutinosa*, located along a latitudinal gradient, to two different temperature treatments, to disentangle the distribution of genetic variation in timing of bud burst and bud burst plasticity within and among genotypes, populations, and regions. We calculated heritability and evolvability estimates for bud burst and bud burst plasticity and assessed the influence of divergent selection relative to neutral drift. We observed higher levels of heritability and evolvability for bud burst than for its plasticity, whereas the total phenological heritability and evolvability (i.e. combining timing of bud burst and bud burst plasticity) suggest substantial evolutionary potential with respect to phenology. Earlier bud burst was observed for the low-latitudinal populations than for the populations from higher latitudes, whereas the high-latitudinal populations did not show the expected delayed bud burst. This countergradient variation can be due to evolution towards increased phenological plasticity at higher latitudes. However, because we found little evidence for adaptive differences in phenological plasticity across the latitudinal gradient, we suggest differential frost tolerance as the most likely explanation for the observed phenological patterns in *A. glutinosa*.

Introduction

The discovery of putatively adaptive associations between genetic and environmental variation is a key objective in evolutionary ecology, as it allows evaluating evolutionary processes with important ecological consequences (Nielsen, 2005; Ingvarsson & Street, 2011). More specifically, the ability of populations to respond to environmental changes through genetic adaptation and phenotypic plasticity influences the genetic composition and dynamics of local populations (Nicotra *et al.*, 2010; Hoffmann & Sgrò, 2011), which may in turn affect ecosystem functioning and resilience (Luck *et al.*, 2003; Whiles *et al.*, 2006; Harmon *et al.*, 2009; Donohue *et al.*, 2013). Tree species in particular play a crucial role in ecosystem functioning, as numerous other species rely on them for food, shelter and nesting potential. Moreover, water and CO_2 regulation, nutrition and wood constitute indispensable ecosystem services provided by tree species. To preserve these services, it is important to investigate the relative contribution of genetic vs. plastic responses of tree populations to adaptive phenotypic variation, and to manage genetic variation within tree populations, allowing them to adapt to future environmental conditions.

Phenotypic plasticity allows populations to respond within a generation to alternating environmental cues (Sultan, 2000; Valladares *et al.*, 2006). This may be

Correspondence: Hanne De Kort, Plant Conservation and Population Biology, Biology Department, University of Leuven, Kasteelpark Arenberg 31, B-3001 Heverlee, Belgium.
Tel.: +32 (0)16 32 15 20; fax: +32 (0)16 32 19 68;
e-mail: hanne.dekort@bio.kuleuven.be

particularly beneficial for tree species, as they are often characterized by (i) long life spans, therefore encountering temporal environmental heterogeneity; (ii) long generation times, which slows down reproduction and genetic responses; (iii) relatively long-distance seed dispersal, increasing the offspring's likelihood of experiencing a different environment; and (iv) efficient gene flow, both through pollen and through seed, which may constrain local adaptation if the homogenizing effects of gene flow exceed the differentiating effects of selection pressure (Kramer, 1995; Aitken *et al.*, 2008).

Plasticity itself can evolve if sufficient additive genetic variation and environmental selection pressure are present (Nussey *et al.*, 2005; Bradshaw, 2006; Lande, 2009; Bijlsma & Loeschcke, 2012). Natural selection for increased plasticity at high latitudes allows quick and intense responses to temperature changes, likely to compensate for the short growing season (Conover & Schultz, 1995; Yamahira & Conover, 2002; Yuan *et al.*, 2011). The ecological and evolutionary consequences of such seasonality-dependent latitudinal compensation have often been studied in ectotherm animal species (e.g. Lisa *et al.*, 2011; Mitchell *et al.*, 2011; Gaitán-Espitia & Nespolo, 2014), but have received surprisingly little attention in plant species. Nevertheless, for tree species from high latitudes, plasticity in vegetative phenology is expected to substantially support population persistence, as the timing of phenological events shapes the critical balance between competition (facilitated by a longer growing season) and avoidance of frost damage (facilitated by a shorter growing season). Analogously, Vitasse *et al.* (2013) found small but significant population divergence for timing of bud burst in five of seven tree species sampled along an altitudinal gradient, as well as small but significant population divergence for bud burst plasticity in four of the tree species. Yet, except for the work of Vitasse *et al.* (2013), few studies attempted to uncover the role of evolution in leaf phenological plasticity in tree species (but see Kramer, 1995). Furthermore, whereas several studies have estimated the amount of phenotypic plasticity by means of reaction norms (e.g. Delpuech *et al.*, 1995; Sultan, 2001; Nussey *et al.*, 2005; Vitasse *et al.*, 2010), these studies rarely accounted for the effects of genetic drift, and therefore may not accurately assess the contribution of natural selection to the evolution of phenotypic plasticity. Yet, neutral processes often affect the quantitative genetic variation among populations for many traits (Lande, 1976; Hodgis-Davis & Townsend, 2009), as has been concluded repeatedly (Merilä & Crnokrak, 2001; McKay & Latta, 2002; Leinonen *et al.*, 2008; De Kort *et al.*, 2012).

In addition to adaptive genetic divergence for phenology and phenological plasticity, substantial genetic variance and adaptive potential can be expected for these traits, as is generally observed for important, fitness-related traits, including bud burst (e.g. Tsarouhas *et al.*, 2003; Alberto *et al.*, 2011; Olson *et al.*, 2013). Indeed, fitness traits have often been found to harbour high quantitative genetic variance and heritability despite strong selection pressures and subsequent adaptive divergence, potentially as a result of balancing selection on adaptive genetic diversity to secure long-term population fitness (Mojica *et al.*, 2012; Alonso-Blanco & Méndez-Vigo, 2014; El-Soda *et al.*, 2014). Yet, despite the potential consequences of phenological plasticity on individual fitness and population persistence, measures of adaptive potential for phenological plasticity traits are hitherto lacking.

The general objective of this study was to estimate the relative role of adaptive evolution, plasticity and the evolution of phenological plasticity in patterns of phenological variation, while explicitly accounting for genetic drift, in the wind-pollinated widespread tree species *Alnus glutinosa*. In a cutting experiment, genetically identical cuttings (clones) from 84 saplings (genotypes), originating from 12 European populations, were grown under two temperature treatments to uncover genetic differences in the magnitude of phenotypic plasticity in bud burst along a latitudinal gradient, and to estimate heritability and evolvability for phenology and plasticity. Our specific research questions were as follows: (i) How is the observed variation in bud burst in *A. glutinosa* distributed among regions, populations, genotypes, clones in different temperature treatments? (ii) Do bud burst and plasticity for bud burst show adaptive divergence along a latitudinal gradient? and (iii) Is the magnitude of bud burst plasticity heritable and evolvable?

Materials and methods

Study species

Black alder (*Alnus glutinosa* (L.) Gaertn, Betulaceae) is a widespread deciduous tree distributed across Europe, from southern Italy up to northern Sweden (Meusel *et al.*, 1965). The species is monoecious, self-incompatible and wind-pollinated, and its seeds are mainly dispersed by water (MacVean, 1953; Chambers & Elliott, 1989). *Alnus glutinosa* is a typical water-demanding species, generally occurring on wetlands and along rivers. The species can fix atmospheric nitrogen in symbiotic root nodules (Bond *et al.* 1954) and therefore does not set high demands on soil nutrition content. This renders the species an excellent candidate for restoring moist degraded soils where it has a good potential for timber production (Claessens *et al.*, 2010). Moreover, *A. glutinosa* is characterized by very high growth rates during the first 20 years, and the wood is very suitable for joinery, energy, fibre for paper, and underwater constructions (Claessens *et al.*, 2010). The species contributes particularly to riverine ecosystems, by providing habitats for a specific flora and fauna both

on the tree itself and in the flooded root system (Dussart, 1999). Finally, the root system supports flood control and stabilizes riverbanks (Boer & Oosterbaan, 2005).

Cutting experiment

A total of 12 populations × 7 individuals × 8 cuttings = 672 cuttings were used for the cutting experiment. The cuttings were taken in early February 2014 from 3-year-old potted saplings that were raised in a previously established outdoor common garden (Geraardsbergen, Belgium, see De Kort *et al.*, 2014), and kept in a cool and dark environment until potting to fulfil the species chilling requirements (Heide, 1993) and to prevent early bud burst. The 12 populations were distributed across a latitudinal gradient comprising four geographical regions (Table 1, De Kort *et al.*, 2014), with temperatures of origin increasing from Sjælland (Denmark), over Flanders (Belgium) and Picardy (France), to Tuscany (Italy) (Fig. S1). In early March 2014, the cuttings were potted and subjected to two temperature treatments (four cuttings in each temperature treatment), using a fully randomized design. An unheated glasshouse compartment was used to simulate the cool environment (mean temperature of 7.5 °C at night and 17.5 °C during the day, corresponding to the Flemish weather conditions), whereas a heated glasshouse compartment adjacent to the unheated compartment provided a warm environment (mean temperature of 15 °C at night and 25 °C during the day, corresponding to the Tuscan weather conditions). The use of standardized conditions in each compartment should minimize confounding of temperature effects by potential compartment effects. The timing of bud burst was recorded by scoring the phenological state of the plants every second day from March to May 2014. We defined bud burst as the time when the leaf tip appeared from the two most apical buds (stage 09 according to the BBCH scale for the phenological growth stages of woody species (Finn *et al.*, 2007)), and the average burst timing of the two buds was used for analyses. Bud burst scoring started with the first bud burst measurement. To limit microenvironmental variation, we kept the cuttings of each treatment in a small area of *ca*. 3 m² for each of the temperature treatments and shuffled the locations of the plants every second day.

Cloning effects, that is phenotypic variation associated with vegetative propagation due to inequalities between the clones, may bias estimates of genetic variance especially in early stages of development (Libby & Jund 1962). Cloning effects can, for example, be caused by variation in the position of the cutting on the donor plant (topophysis), or by unequal treatment of the clones during cutting and potting. By estimating the environmental variance based on multiple clonal replicates within genotypes, cloning effects can be partially accounted for (Libby & Jund 1962). We also measured the mass of each clone to control for dissimilarities among clones.

SNP genotyping

The 84 maternal genotypes were part of a larger panel of individuals that were genotyped in a previous study to analyse SNPs in a landscape genomic context (see Table 1 for the number of genotypes per population). This landscape genomic study revealed a dominant role for temperature in explaining genomic and phenotypic patterns (De Kort *et al.*, 2014). Briefly, DNA was extracted using DNeasy Plant Extraction kits (Qiagen Inc., Valencia, CA, USA), at a concentration of approximately 100 ng μL^{-1}, as measured using a NANODROP2000 spectrophotometer (ISOGEN LIFE SCIENCE, Belgium). DNA integrity was evaluated on 1.5% agarose gels (De Kort *et al.*, 2014). De novo genotyping-by-sequencing (GBS) was used for constructing reduced representation *PstI* libraries for the Illumina HiSeq 2000 (Elshire *et al.*, 2011). Raw DNA sequences were analysed with the Universal Network Enabled Analysis Kit (UNEAK) pipeline, implemented in TASSEL v3.0 (Lu *et al.*, 2013). The resulting genotypes were filtered to those with sequencing depth between 3 and 127 (using VCFtools v0.1.10; Danecek *et al.*, 2011). Final filtering of the data involved elimination of individuals with greater than 90 % missing data and SNPs with more than 20 % missing data (VCFtools v0.1.10). Overall, this yielded genotypes at 1990 polymorphic loci (a total of 31 monomorphic loci were excluded) with on average 3.47 % missing data per genotype (see De Kort *et al.*, 2014 for a more detailed description of the GBS protocol). Of these 1990 SNPs, 57 SNPs (2.86%) were detected as outliers and may therefore not represent neutral genetic processes, whereas the other 1933 SNPs were considered neutral. We previously showed that mean population heterozygosity (H_E) ranged between 0.237 and 0.260, and a principal coordinates analysis (PCoA) revealed 2 distinguishable genetic groups (the Italian populations vs. all other populations) (Table 1, Fig. S2; De Kort *et al.*, 2014).

Variation in bud burst among clones, genotypes, populations and regions

As an exploratory analysis, a hierarchical mixed-effects model was built to estimate the relative distribution of the variation in bud burst (Y) among regions ($\alpha_{i,}$ random effect), among populations nested within regions ($\beta_{j(i),}$ random effect), among genotypes nested within populations and regions ($\gamma_{k(j(i)),}$ random effect), and among clones within genotypes (eqn 1) ('residual'). 'Mass' (δ_{ijkl}) was introduced as a fixed covariate to control for potential effects of clone mass on timing of bud burst.

Table 1 Population-specific information on geography, climate (bioclim 1 from Worldclim.org representing yearly mean temperatures in °C), sample size and heterozygosity. N_{gen} reflects the number of samples used for the neutral genetic analyses in this study. Data adopted from De Kort *et al.* (2014).

Population	Region	Latitude	Longitude	T	N_{gen}	H_e
De Pinte	Flanders	50.983930	3.629963	10.2	13	0.253 (±0.004)
Evergem	Flanders	51.165980	3.681092	10.2	14	0.259 (±0.004)
Moerbeke	Flanders	51.145547	3.918045	10.3	5	0.229 (±0.005)
Boves	Picardy	49.856944	2.378333	10.4	13	0.251 (±0.004)
Saint-Michel	Picardy	49.947500	4.212778	8.9	11	0.257 (±0.004)
Hanappes	Picardy	49.984167	3.596389	9.4	17	0.259 (±0.004)
Casina Rossa	Tuscany	43.165054	11.220419	14.0	12	0.254 (±0.004)
Famelunga	Tuscany	43.121808	11.176541	13.7	14	0.247 (±0.004)
Tocchi	Tuscany	43.136084	11.254197	13.3	13	0.239 (±0.004)
Gundsømagle Sø	Sjælland	55.726750	12.194283	8.4	15	0.253 (±0.004)
Lyngby Åmose	Sjælland	55.777750	12.483417	8.3	14	0.246 (±0.004)
Borrevejle Skov	Sjælland	55.645683	11.929267	8.4	11	0.238 (±0.004)

$$Y_{ijkl} = \mu + \alpha_i + \beta_{j(i)} + \gamma_{k(j(i))} + \delta_{ijkl} + \varepsilon_{ijkl} \quad (1)$$

We repeated the mixed-effects modelling for each of both temperature treatments, which allowed us to visualize how the contribution of the different hierarchical levels to the total genetic variance varies among temperature treatments. We $\log_{10}$-transformed the timing of the bud burst to comply with the assumption of normality.

Assessing the degree of adaptive divergence

Because genetic differences in bud burst and its plasticity among populations may partially result from neutral processes, we followed the Bayesian approach of Ovaskainen *et al.* (2011) to statistically assess the degree of local adaptation. This method offers an improved alternative for the traditional Q_{ST}-F_{ST} approach (Wright, 1951; Spitze, 1993), by controlling for (i) genetic nonindependence among populations (co-ancestry), and (ii) the randomness that is inherent to the evolutionary process. We first applied the admixture F-model (Karhunen & Ovaskainen, 2012) to estimate the population-level co-ancestry matrix θ^P from the neutral SNP data. We then applied the quantitative genetics model of Ovaskainen *et al.* (2011) to estimate the amount of additive genetic variance in the common ancestral population (V_A), the amount of environmental variance (V_E), and the vector of population mean genetic values (a^P). Temperature treatment (cool vs. warm) was included as fixed effect to measure the overall plastic response of bud burst to temperature. The posterior distributions of the model parameters (a^P, θ^P, V_A, V_E) were estimated with the Bayesian estimation scheme R-package driftsel (Karhunen *et al.*, 2013), modified to account for the clonal structure in the data.

Under neutral genetic drift, the pattern of population divergence would follow the distribution (Ovaskainen *et al.*, 2011).

$$a^P \sim N(0, 2V_A\theta^P). \quad (2)$$

To examine whether the realized pattern showed regionally structured or a temperature-related signal of local adaptation, we applied a slightly modified version of the H-test by Karhunen *et al.* (2014). As a test statistic, we used the proportion of variance (R^2) among the population means that is explained either by the region (categorical variable) or by temperature of the population origins (continuous variable). We computed the posterior probability of the observed proportion of variance being greater than the neutral expectation based on eqn 2. We $\log_{10}$-transformed the timing of the bud burst to comply with the assumption of normality.

To look for a signature of selection for plasticity, we repeated the same approach for bud burst plasticity (P), which was calculated as the timing of bud burst in the cool treatment (BBc) *minus* the timing of bud burst in the warm treatment (BB_W), with *ca.* four measurements per genotype (four clones per temperature treatment).

Estimating current evolutionary potential

Broad-sense heritability (H^2) of bud burst and bud burst plasticity was estimated as the proportion of the total phenotypic variance that can be attributed to genetic variance $[\sigma^2_{gen}/(\sigma^2_{gen} + \sigma^2_e)]$, with σ^2_{gen} representing the variance among genotypes, and σ^2_e reflecting the residual variance that is due to environmental effects (Falconer & Mackay, 1996; Volis *et al.*, 2005). We ran this random-effects model using the program H2boot (subroutine for clonal lines; Phillips, 1998; Phillips & Stevan, 1999; with 10 000 bootstrap samples. The populations of *A. glutinosa* inhabiting the same region are genetically similar (Fig. S2) and experience similar temperature conditions. Therefore,

heritability leaf phenological responses to variation in temperature were estimated at the level of the region and, in the case of bud burst, for each temperature treatment.

As quantitative traits such as phenological traits have complex genetic architectures, covariances between environmental and genetic effects may cause unreliable heritability estimates (Houle, 1992; Hansen *et al.*, 2003; Wilson, 2008; Hemani *et al.*, 2013; Nespolo *et al.*, 2013). Complex traits such as leaf phenology are underpinned by a genetic architecture involving many potential targets that can be simultaneously affected by genetic and environmental perturbations (Hansen *et al.*, 2011). This can result in a correlation between genetic and environmental variance, thereby biasing classical heritability measurements that assume environmental variance to be independent from genetic variance. We therefore also calculated the broad-sense evolvability, which is a mean-scaled measure of genetic variance and therefore independent of other sources of variance (Hansen *et al.*, 2003, 2011; Garcia-Gonzalez *et al.*, 2012). Evolvability (E) was calculated using a random-effects ANOVA as σ^2_{gen}/m^2, with m representing the trait mean. This measure reflects the expected proportion of change per generation for traits under a unit strength of selection (Hansen *et al.*, 2003, 2011).

Results

Variation in bud burst among clones, genotypes, populations and regions

Average timing of bud burst increased from Tuscany (3.33 ± 0.24 days after first observation) over Sjaelland (4.89 ± 0.32 days) to Picardy (6.15 ± 0.31 days) and Flanders (6.16 ± 0.35 days), and from the warm (4.56 ± 0.16 days) to the cool environment (6.65 ± 0.25 days). Surprisingly, the populations from Sjaelland did not show the delay in bud burst, as is expected based on its latitudinal position (Fig. 1).

The mixed-effect models revealed that most of the bud burst variance occurred among clones of the same genotype (40.03% and 60.75% in the warm and the cool treatment, respectively) and among genotypes within populations (40.34% and 24.63% in the warm and the cool environment, respectively). Also 'region' explained part of the bud burst variance (19.62% and 14.62% in the warm and the cool environment, respectively), whereas no variance was attributed to differences among populations in the same region (Fig. 2). Temperature clearly affected the amount of variance in bud burst explained by 'genotype' as well as the residual variance within genotypes (Fig. 2), indicating genotype- and clone-dependent plasticity. Cutting mass did not explain a significant amount of genetic variance in bud burst (3.01%, $P > 0.05$; and 2.31%, $P > 0.05$, respectively).

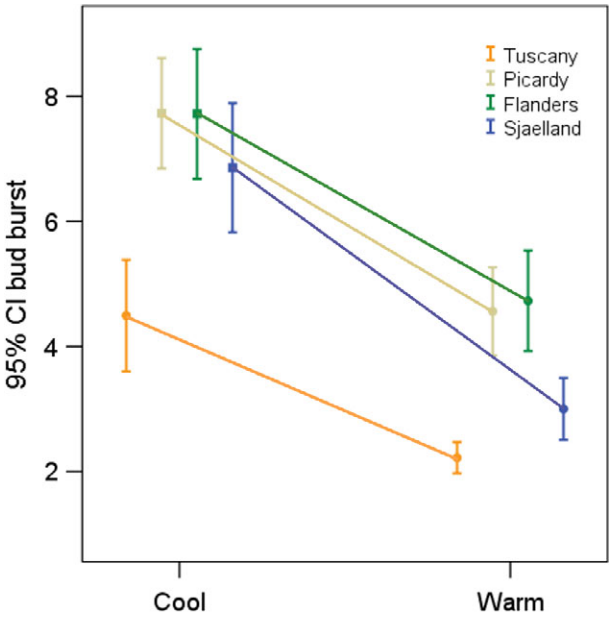

Fig. 1 Timing of bud burst (with 1 the day of the first bud burst measurement, 25 March) as a function of temperature treatments (reaction norms) for the four regions. Confidence intervals for means represent genetic and environmental variation within genotypes, populations and regions. Within temperature treatment, the regions are ordered from low to high latitude.

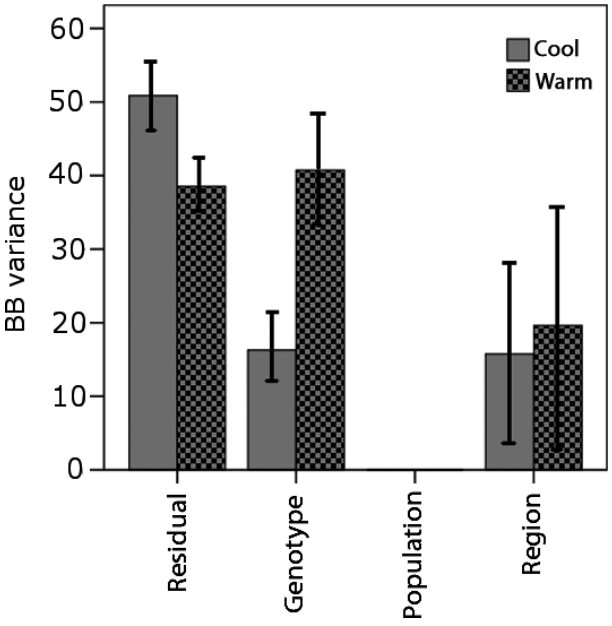

Fig. 2 Percentage variance (with standard error) in bud burst (log_{10}-transformed) explained by regional differences ('region'), population differences ('population(region)') and genotype differences ('genotype(population(region))'), and residual variance (within genotype variation). The bud burst variance includes both genetically and environmentally induced variance.

Assessing the degree of adaptive divergence

The posterior probabilities (H) for divergent selection with respect to the temperature gradient and with respect to region were 0.77 ($R^2 = 0.21$) and 0.86 ($R^2 = 0.50$), respectively, thus yielding only low-to-moderate evidence for adaptive variation in bud burst (Table S1). Excluding the Sjaelland populations, which did not show the expected delay in bud burst, from the analyses resulted in a substantially higher posterior probability for divergent selection imposed by the temperature gradient ($H = 0.93$, $R^2 = 0.46$, Table S1).

We found clear evidence for the presence of plastic responses to temperature (Figs 1 and 2, Table S1). The posterior median value for the effect of temperature treatment was 0.27 (95% credibility intervals 0.23–0.30). However, estimated population means for plasticity only slightly varied between Tuscany (−0.27 to −0.05), Picardy (−0.22 to 0.10), Flanders (−0.38 to 0.00) and Sjaelland (−0.03 to 0.19; Table S1). Correspondingly, no divergent selection was observed among populations or along the temperature gradient ($H = 0.51$ and $H = 0.52$, respectively), implying limited adaptive divergence of bud burst plasticity. Although the Danish populations showed some indications for increased plasticity (Fig. 1), this trend was not significant after accounting for both the phenotypic variation among clones within genotypes and the neutral genetic component.

Estimating current evolutionary potential

Broad-sense heritability for bud burst varied between 0.139 (Picardy, cool) and 0.540 (Tuscany, warm), whereas evolvability for bud burst ranged between 0.032 (Picardy, cool) and 0.190 (Picardy, warm) (Fig. 3, Table 2). Tuscany and Picardy had higher H^2 and E in the warm treatment, whereas Flanders and Sjaelland had higher H^2 and E in the cool treatment. For bud burst plasticity, broad-sense heritability varied between 0.000 (Picardy) and 0.129 (Sjælland), and evolvability between 0.000 (Picardy) and 0.256 (Tuscany) (Fig. 3, Table 2).

Discussion

In this study, we dissected the phenotypic variation in bud burst in a common tree species into multiple components: (i) genetic differentiation of bud burst reflecting adaptive phenological evolution, (ii) bud burst plasticity, and (iii) genetic differentiation of bud burst plasticity, reflecting adaptive divergence of plasticity. This was achieved through subjecting clones of saplings to different temperature treatments. In descending order, variance in bud burst was attributed to maternal variance within genotypes, genetic variance among genotypes, and genetic variance among regions (see Fig. 2). Northern populations tended to be more plastic than mid- and low-latitudinal populations with respect to bud burst, yet, in contrast to bud burst, no footprint of selection was found for bud burst plasticity. Heritability and evolvability estimates varied considerably among temperature treatments (for bud burst) and among regions (for bud burst and bud burst plasticity). On overall, our findings suggest high potential to adapt phenology (combining bud burst and bud burst plasticity) to future environmental conditions.

Bud burst plasticity within and between genotypes

Remarkably, most of the variance in timing of bud burst resided among clones. Considering that bud burst measurements were accurate and the effect of cutting mass on bud burst limited, this suggests a role for epigenetic control. The same genotype may indeed harbour different epigenetic imprints, which can alter gene expression and phenotypic values accordingly (e.g. DNA methylation) (Wong *et al.*, 2005; Verhoeven *et al.*, 2010; Verhoeven & Preite, 2014). The involvement of epigenetic mechanism during bud burst and bud set has been demonstrated repeatedly (Santamaría *et al.*, 2009; Conde *et al.*, 2012; Yakovlev *et al.*, 2012). In *Castanea sativa*, for example, DNA methylation patterns differed between apical buds, which flushed earlier, and axillary buds, which flushed later or not at all (Santamaría *et al.*, 2009). Because in our study genetically identical cuttings were taken from different

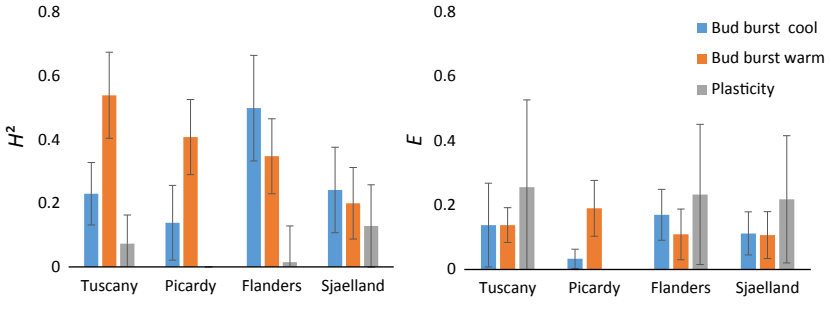

Fig. 3 Broad-sense heritability (H^2) and evolvability (E) with error bars of bud burst and plasticity for the four European regions. Regions are ordered from low to high latitude.

Table 2 Variance components (*$P < 0.05$, ***$P < 0.001$), broad-sense heritability (H^2) and evolvability (E) of bud burst and bud burst plasticity with standard errors for the four European regions, ordered from low to high latitude. Bud burst values were calculated for both the cool and the warm treatment.

Trait	Region	V_{gen}	V_{res}	Mean	H^2	E
Bud Burst (cool)	Tuscany	2.490	12.184***	4.494	0.230 (±0.098)	0.138 (±0.130)
	Picardy	1.902	12.518***	7.730	0.139 (±0.118)	0.032 (±0.030)
	Flanders	8.500*	8.173***	7.720	0.499 (±0.166)	0.170 (±0.079)
	Sjælland	5.268	16.605***	6.858	0.242 (±0.134)	0.112 (±0.067)
Bud Burst (warm)	Tuscany	0.667*	0.604***	2.220	0.540 (±0.135)	0.135 (±0.054)
	Picardy	3.943*	5.355***	4.561	0.408 (±0.118)	0.190 (±0.087)
	Flanders	2.768	7.426***	4.729	0.348 (±0.118)	0.109 (±0.079)
	Sjælland	0.955	4.043***	3.000	0.200 (±0.113)	0.106 (±0.073)
Plasticity	Tuscany	1.450	11.093***	2.467	0.073 (±0.090)	0.256 (±0.271)
	Picardy	0.000	19.459***	3.286	0.000 (±0.100)	0.000 (redundant)
	Flanders	1.412	11.299***	2.100	0.015 (±0.113)	0.233 (±0.218)
	Sjælland	3.858	20.990***	3.858	0.129 (±0.130)	0.220 (±0.198)

positions on the same branch, the observed variation within genotypes may be the consequence of position-driven epigenetic mechanisms (topophysis) that were already present in the sapling of origin ('maternal' plasticity). We cannot fully exclude a potential role of microenvironmental variations or cloning effects on differences among clones, but nevertheless we suspect a more important effect of epigenetic mechanisms given the limited space in which the clones were raised and the limited effects of mass on variance among clones in the same temperature treatment and within genotypes. Another large part of the bud burst variance was observed among genotypes (within identical temperature treatment), which is generally assumed to result from genetic differences (Fig. 2). However, this assumption may be overrated in many common garden studies. Indeed, we observed a strong effect of the local temperature on the variance among genotypes (Fig. 2), indicating that at least part of the genetic trait differentiation inferred from common garden experiments can be biased considerably by phenotypic variation (epigenetically) induced or removed by the common garden environment (see also Gienapp *et al.*, 2008; De Kort *et al.*, 2014). This variance component can be taken into account by examining trait responses of genotypes originating from different populations in different environmental conditions.

Adaptive evolution of bud burst and bud burst plasticity

A total of 14.6% of the bud burst variance was attributed to differences among regions, suggesting adaptive trait divergence. The Bayesian test for selection, which accounts for neutral genetic variation and co-ancestry, provided additional support for the occurrence of natural selection imposed by a temperature gradient driving genetic differences in the timing of bud burst. However, although we expected delayed bud burst for the cuttings from Sjælland (Denmark) due to shorter growing season, we observed rather the opposite pattern, with earlier bud burst than for saplings originating from the mid-latitudinal regions (Fig. 1). Such countergradient variation, where phenotypic and genetic gradients show opposite responses to environmental variation, has been reported for only a few tree species, including several temperate conifer species and *Fagus sylvatica* (Vitasse *et al.*, 2009; Alberto *et al.*, 2013; Kremer *et al.*, 2014). Countergradient variation is generally thought of as being the result of plastic and genetic response acting antagonistically and, in the case of bud phenology, has been suggested to reflect different compromises in the evolutionary trade-off between exposing new leaves to late frost and maximizing the growing season length (Alberto *et al.*, 2013; Kremer *et al.*, 2014). The observed countergradient pattern is in line with the expectation that high-latitudinal populations can compensate for the short growing season by increased phenotypic sensitivity to temperature changes in spring (Yuan *et al.*, 2011). In *A. glutinosa*, the countergradient variation at high latitudes may be associated with (i) slightly increased plasticity for bud burst, as the Sjælland region showed the steepest reaction norm (Fig. 1), and (ii) potential interactions between phenology and unmeasured traits such as frost tolerance. Correspondingly, previous common garden research (in Pennsylvania) observed relatively high frost tolerance in *A. glutinosa* offspring originating from Denmark, combined with relatively early bud burst (DeWald & Steiner, 1986). This suggests that both physiological and phenological parameters are necessary to explain adaptive differences in bud burst according to a latitudinal gradient. Whereas the high-latitudinal populations showed countergradient variation, this was not the case for the low-latitudinal populations, indicating non-monotonic responses to a latitudinal gradient. A

theoretical study recently suggested that nonmonotonic responses may be the rule rather than the exception due to the common interplay between physiological, genetic and demographic effects (Oddou-Muratorio & Davi, 2014).

The lack of adaptive differentiation in plasticity as compared to mean trait value may imply limited selective pressure imposed by current local environmental heterogeneity. Additional research on the relative contribution of genetic and plastic responses to environmental change could benefit from a higher number of clones per genotype, larger population sample sizes, and treatments simulating the climate experienced by all studied populations, which (i) may render more robust results and (ii) would allow integrating reaction norms in classical models predicting bud burst dates (Arora & Boer, 2009; Bennie *et al.*, 2010).

Evolvability of leaf phenology and plasticity

Heritability for bud burst was moderate (0.14–0.54), and lower than values estimated for other deciduous species (e.g. H^2 = 0.21–0.47 for *Populus balsamifera*, Farmer, 1993; h^2 = 0.55–0.83 for *Robinia pseudoacacia*, Mebrahtu & Hanover, 1989; h^2 = 0.87 for *Quercus petraea*, Alberto *et al.*, 2011). This moderate heritability suggests limited potential to evolve new trait values that support fitness in novel environmental conditions (Mousseau & Roff, 1987; Bolnick *et al.*, 2011). However, heritability varied considerably among populations and temperature treatments (Table 2, Fig. 3), with relatively high values for the populations in the temperature treatment resembling the climate of the populations' origins. More specifically, Flemish populations showed highest heritability in the cool environment, whereas Tuscan populations showed highest heritability in the warm environment due to lower environmental variance (Table 2). Whereas low heritabilities in unfavourable conditions are not uncommon for morphological traits because suboptimal growth conditions may constrain evolutionary potential (see Hoffmann & Shiffer, 1998; Hoffmann & Merilä, 1999; Charmantier & Garant, 2005), our finding suggest that this relation also holds for phenological traits. With respect to plasticity, the populations from Sjælland showed highest heritability, suggesting that these populations are capable of evolving different magnitudes of plasticity depending on the reliability and heterogeneity of future temperature conditions. Overall, the limited phenological heritability in *A. glutinosa* in concert with a limited signature of adaptive divergence may indicate some genetic constraints on adaptive evolution as a result of genetic correlations between traits under positive selection and traits subject to antagonistic selection (Weinreich *et al.*, 2005; Futuyma, 2010). On the other hand, given the environment-dependent nature of heritability estimates, the rather moderate pattern of adaptive divergence may also suggest that selection pressures are limited, but that evolutionary potential may increase with changing selection regimes (Sgro & Hoffmann, 2004; Gienapp *et al.*, 2008; Bonduriansky *et al.*, 2012).

We observed moderate estimates of evolvability for bud burst (0.03–0.19%) and for bud burst plasticity (0.00–0.26%). These values are similar to median estimates for morphological traits, which predict 0.1% change per generation for traits under unit selection (Hansen *et al.*, 2011). Hence, depending on the strength of selection, bud burst and especially plasticity for bud burst may evolve relatively quickly. For example, with the percentage of change over t generations being $(1 + E\beta)^t$ (Hansen *et al.*, 2011), and with a selection strength β of 0.3 (Hereford *et al.*, 2004) and an evolvability E of 0.2%, the trait value can double in approximately 12 generations (ca. two centuries for *A. glutinosa*). These estimates are in line with simulations showing that under strong natural selection and considerable gene flow, quantitative traits may evolve quickly in the first generations due to inheritance of beneficial allelic combinations (Le Corre & Kremer, 2012). Moreover, although the relative contribution of bud burst and bud burst plasticity to the total evolvability greatly varied among regions and temperature treatments, all regions seem to harbour substantial evolutionary potential when taking into account the evolvability of both phenological traits (Fig. 3).

Finally, the observed variation in plastic (i.e. nongenetic) responses of bud burst to temperature (Fig. 2) and the differences in evolvability of the same genotypes among temperature treatments (Table 2) may suggest that the evolvability of phenology involves an epigenetic component (Danchin *et al.*, 2011; Zhang *et al.*, 2013). The potential to inherit nongenetic phenotypic variation has been demonstrated repeatedly (e.g. Verhoeven *et al.*, 2010; Crews *et al.*, 2012; Zhang *et al.*, 2013) and may have important evolutionary consequences as it allows to evolve traits more rapidly and flexibly in function of changing environments (Richards, 2006; Danchin *et al.*, 2011; Bonduriansky *et al.*, 2012).

Conclusions

The successful balancing of phenological events and their underlying molecular basis has important implications for the dynamics and persistence of tree populations and for associated biotic interactions at the community level. The use of clones allowed disentangling the contribution of evolution and plasticity of bud burst within and among genotypes, populations and regions. Our study revealed limited evolution of timing of bud burst and bud burst plasticity, with their relative importance depending on the latitude of origin. Despite the moderate heritability and evolvability

for bud burst and plasticity observed in our study, their combined effects may suggest considerable capacity to evolve phenology in response to climate change. Although our results are indicative for adequate evolutionary potential for phenology, additional experimental studies are needed to uncover the molecular basis of phenological plasticity, and to predict responses to projected future climate conditions (Neale & Kremer, 2011; Wolkovich *et al.*, 2014). Nevertheless, our results deliver insights into the evolutionary potential of *A. glutinosa* to cope with environmental change, through both genetic and plastic responses, and suggest substantial evolvability provided that management continues to support genetic exchange among populations.

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Data accessibility

Raw read data are available for download from SRA using the following accession number: SRS595548.

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Supporting information

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

Figure S1 Results of a principal components analysis (PCA) on the climate variables (Temperature and Precipitation), and temperature (PC1) of the regions included in our study.

Figure S2 Plots of a principal coordinates analysis (PCoA) on the genetic distances between the sampled individuals based on 1990 SNPs.

Table S1 Data set and raw results of the Bayesian test for selection.

Data deposited at Dryad: doi: 10.5061/dryad.rg82f

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