

Natural selection and climate change: temperature-linked spatial and temporal trends in gene frequency in *Fagus sylvatica*

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Abstract

Rapid increases in global temperature are likely to impose strong directional selection on many plant populations, which must therefore adapt if they are to survive. Within populations, microgeographic genetic differentiation of individuals with respect to climate suggests that some populations may adapt to changing temperatures in the short-term through rapid changes in gene frequency. We used a genome scan to identify temperature-related adaptive differentiation of individuals of the tree species *Fagus sylvatica*. By combining molecular marker and dendrochronological data we assessed spatial and temporal variation in gene frequency at the locus identified as being under selection. We show that gene frequency at this locus varies predictably with temperature. The probability of the presence of the dominant marker allele shows a declining trend over the latter half of the 20th century, in parallel with rising temperatures in the region. Our results show that *F. sylvatica* populations may show some capacity for an *in situ* adaptive response to climate change. However as reported ongoing distributional changes demonstrate, this response is not enough to allow all populations of this species to persist in all of their current locations.

Keywords: adaptation, AFLP, climate change, genome scan, natural selection, population genomics

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Introduction

Recent reviews of the evolutionary effects of climate change have highlighted the potential for climatic warming to cause strong directional selection in natural populations (Jump & Peñuelas 2005; Thomas 2005). Climate is a key factor determining the distribution of plant species (Woodward 1987); the frequent differentiation of plant populations with respect to climate demonstrates that climate exerts strong selective pressure on natural populations (Turesson 1925; Clausen *et al.* 1940; Linhart & Grant 1996; Joshi *et al.* 2001; Sackville Hamilton *et al.* 2002; Etterson 2004). One of the basic assumptions in the study of plant adaptation to environment (genecology) is that natural selection in different environments generates genetic clines that correlate with environmental clines. This is well illustrated by recent pan-European studies of

local adaptation in common plant species (Joshi *et al.* 2001; Sackville Hamilton *et al.* 2002). Geographic patterns in neutral genetic diversity and population genetic structure may be generated during species' migrations (Hewitt 2000; Comps *et al.* 2001). However, temperature is of major importance as a selective agent causing population differentiation over altitudinal and latitudinal clines (Saxe *et al.* 2001). Recent rapid climatic warming represents a strong temporal temperature cline that parallels temperature clines that are geographically based. Temporal changes in gene frequency that result from global warming should therefore mirror spatial changes observed with decreasing altitude and latitude.

Repeated, ecologically correlated patterns of molecular marker diversity have been detected in a variety of species from populations sampled across contrasting habitats. In these studies, particular alleles may be confined to or occur preferentially in, different sites with contrasting environmental conditions. These patterns often occur at both microgeographic and regional scales, and have been reported in

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plant species including the wild cereals *Avena barbata* (Hamrick & Allard 1972; Hamrick & Holden 1979), *Hordeum spontaneum* (Owuor *et al.* 1997, 2003) and *Triticum dicoccoides* (Li *et al.* 1999, 2000), and the trees *Betula pendula* (Kelly *et al.* 2003) *Pinus edulis* (Cobb *et al.* 1994; Mitton & Duran 2004) and *Picea engelmannii* (Stutz & Mitton 1988; Mitton *et al.* 1989). Such environmental correlation of molecular marker diversity is interpreted as evidence of adaptive genetic differentiation (Linhart & Grant 1996; Nevo 2001). The opposing view, based on evidence gained from some of the same species, is that these correlations of alleles with particular environments can be explained by factors other than natural selection, or even by chance alone (Volis *et al.* 2003, 2004). Much of this difference in opinion relates not to whether or not local adaptation occurs, but at what geographic scale, and how best to detect its genetic signature.

Assuming that selection is operating between populations of a species occurring over contrasting habitats, if individuals are screened at a large number of molecular marker loci then it is likely that some of these loci will fall in, or near, selected genes (Luikart *et al.* 2003). Comparison of locus-specific genetic differentiation with the genome-wide average will highlight these loci as outliers with exceptionally high or low differentiation. This population genomic approach can therefore identify those loci bearing the signature of natural selection acting on linked regions of the genome (Vitalis *et al.* 2001; Luikart *et al.* 2003; Beaumont 2005; Storz 2005). The population genomic approach to identifying adaptive genetic differentiation is based on rigorous statistical methods that substantially reduce the problem of false positives (random correlations between allele frequency and the environment) that are associated with the correlative approach outlined above (Nielsen 2001; Vitalis *et al.* 2001; Sackville Hamilton *et al.* 2002; Luikart *et al.* 2003; Beaumont & Balding 2004).

Following the identification of an outlier locus, it is necessary to verify and determine the cause of the outlier behaviour. Additional methods that can point to a causal relationship between environmental variation and genetic differentiation should be used to confirm the action of natural selection (Sackville Hamilton *et al.* 2002; Luikart *et al.* 2003). Such methods include repeated independent population comparisons conducted over the same environmental gradient (Wilding *et al.* 2001), determination of consistent phenotypic differences between the studied populations and mapping of quantitative trait loci (QTL) (Rogers *et al.* 2002; Campbell & Bernatchez 2004; Rogers & Bernatchez 2005), and identification of candidate genes by proximity to selected loci (Vigouroux *et al.* 2002). In non-model organisms however, association or correlation between an allele and a trait or an environmental variable, will often be the best available evidence for adaptive significance of outlier behaviour (Luikart *et al.* 2003). The correlative approach to identifying adaptive genetic differ-

entiation should not therefore be dismissed, but rather used to seek confirmation of outlier behaviour following the identification of candidate loci using population genomic methods.

In this study we combined population genomic and correlative approaches to identify adaptive genetic differentiation linked to temperature within a natural population of the tree species *Fagus sylvatica* L. in the Montseny Mountains of Catalonia, northeastern Spain. By combining amplified fragment length polymorphism (AFLP) molecular markers from a total of 209 trees with dendrochronological data, we tested the hypotheses (i) that temperature extremes experienced at the upper and lower altitudinal limits of the species distribution should result in adaptive differentiation of the trees in these areas; and (ii) that spatial and temporal variation in temperature should lead to predictable variation in gene frequency at the loci under selection. We demonstrate that natural selection by climate has led to locus-specific genetic differentiation in *F. sylvatica* in this region and that directional genetic change has occurred as consequence of warming temperatures over the last half-century.

Materials and methods

Study species, sites and sampling

Fagus sylvatica (European beech) is a monoecious, diploid, late-successional tree that dominates temperate forests over approximately 17 million hectares (ha) of Europe. It is highly outcrossing and largely self-incompatible with irregular synchronous flowering (masting) events. Reproduction does not begin until the species is 40–50 years old (Nilsson & Wastljung 1987; Comps *et al.* 2001).

The Montseny Mountains, where this work was conducted, lie 50 km north–northwest of Barcelona and 20 km inland from the Mediterranean Sea. *F. sylvatica* reaches the southern edge of its distribution in Europe in the Montseny Mountains, occurring in the temperate zone above approximately 1000 m above sea level (a.s.l.) (Fig. 1) and forming approximately 2830 ha of near-continuous forest along the Turó de l'Home (1712 m a.s.l.) and Les Agudes (1706 m a.s.l.) ridge. This species forms the treeline on Turó de l'Home and Les Agudes, above which lies a small area of subalpine vegetation dominated by *Juniperus communis* L. and *Calluna vulgaris* L. The lower limit of *F. sylvatica* typically marks the boundary between the temperate zone and the Mediterranean zone (dominated by *Quercus ilex* L. forest). A detailed description of the vegetation of Montseny and its altitudinal zonation is presented by Bolòs (1983).

We selected three sites within the largest area of continuous forest with no evidence of any recent disturbance. These included the upper treeline spanning Turó de l'Home and Les Agudes (high *Fagus* limit, HFL), an area of

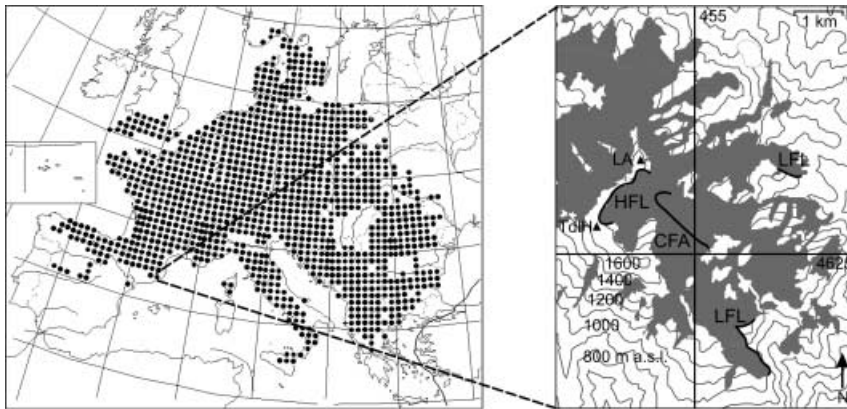


Fig. 1 Left: native distribution of *Fagus sylvatica* (reproduced from Jalas & Suominen (1976), by permission of the Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo). Right: location of study sites in relation to *F. sylvatica* distribution in the study area. *F. sylvatica* distribution is shown in grey. Study sites: HFL upper treeline (high *Fagus* limit); CFA, central forest area; LFL, low *Fagus* limit. Bold lines show the distribution of individuals sampled at each site. Principal peaks are marked with a triangle: TdIH Turó de l'Home; LA Les Agudes. Grid lines are numbered with UTM coordinates in km.

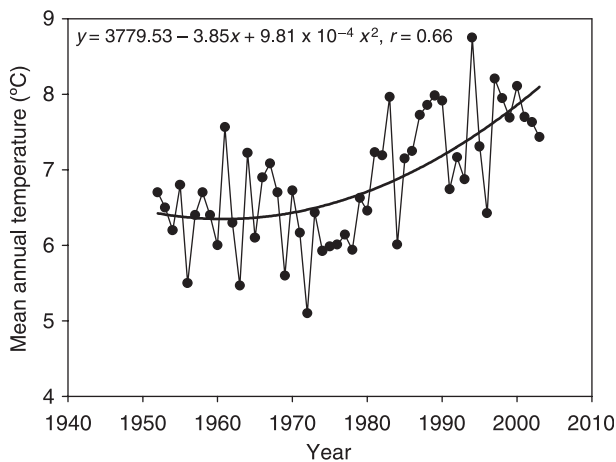


Fig. 2 Mean annual temperature measured at Turó de l'Home meteorological station (1712 m above sea level) with warming trend indicated by the regression line.

the forest interior (central forest area, CFA) and the lower limit of *F. sylvatica* forest (low *Fagus* limit, LFL). All three sites are on the southeast side of the Turó de l'Home-Les Agudes ridge. The sites were separated by a maximum of 5 km horizontal distance with a mean altitude in metres above sea level (m a.s.l.) of 992 for the LFL sample, 1127 for the CFA and 1640 for the HFL (Fig. 1). Mean annual temperature data were available for the period 1952–2003 from the Turó de l'Home meteorological station (1712 m a.s.l.) directly above our HFL study site (Fig. 2).

We sampled 70 juvenile trees at each of the three study sites during spring 2004. These were straight, single-stemmed individuals estimated to be under 40 years old, based on dendrochronological data from a pilot study. Trees were sampled over 2 km of forest, spaced as evenly as establishment patterns allowed. It was problematic to sample forest without recent disturbance at the LFL as forestry plantations and agriculture have a significant impact here. Consequently, it was necessary to split the

LFL site between two sections separated by 3 km at the lower altitudinal limit of *F. sylvatica* (Fig. 1).

For DNA extraction, we sampled newly unfolding leaves or leaf buds from each tree and dried these immediately in fine-grain silica gel. We also took increment cores at a height of 15 cm using a 4.3 mm increment borer. Two or three cores were taken from each tree depending on the quality of the cores and ensuring one core passed through the centre of the tree. Trees that were too small to permit the removal of an increment core were felled at a height of 15 cm and a stem disc removed. Samples were dried, mounted on wood supports and sanded to prepare them for tree-ring analysis using standard dendroecological methods. Prepared samples were then scanned at 1600 d.p.i. using a flatbed scanner and the data saved as JPEG files.

Dendrochronological analysis

Cores and discs from juvenile trees were dated by ring counting using CooRecorder (Larsson 2003). Age at 15 cm was estimated for each core for each tree separately, or from several radii for each stem disc. The modal value of the number of annual terminal bud scars at 15 cm, counted on a sample of 30 saplings at each site, was added to the age at 15 cm to give total age for each juvenile tree. Age estimation accuracy was estimated by comparison of individual ring counts and re-counts made on a random selection of 10% of cores and discs and from the variability of terminal bud counts. We excluded trees that could not be dated and those that established before 1952, the earliest year covered by mean annual temperature data. This left a total of 179 trees (85% of the total) to be used in the temperature and age-based analyses.

AFLP analysis

Dried leaf tissue (0.5 cm²) was ground in liquid nitrogen for 30 s at 30 rps using a mixer mill (Tissue Lyser, QIAGEN) and two glass beads. Genomic DNA was extracted from

ground tissue using a DNeasy Plant Mini Kit (QIAGEN) and quantified using a NanoDrop ND-1000 spectrophotometer running software version 3.0.1 (NanoDrop Technologies), following the manufacturers instructions.

AFLP analysis followed a modified version of the original protocol published by Vos *et al.* (1995), as reported below. Oligonucleotide sequences for polymerase chain reaction (PCR) adapters and primers are those used in the original protocol.

Restriction digests and ligation of adapters. DNA (0.5 µg) was digested at 37 °C for 3.5 h with 5 units *EcoRI* (Roche Applied Science) and 5 units of *Tru9I* (an isoschizomer of *MseI*) (Roche), in 40 µL of buffer comprising 33 mM Tris acetate (pH 7.9 at 37 °C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol (DTT) (SuRE/Cut Buffer A, Roche) and 50 ng/µL BSA.

PCR adapters were ligated to the cut fragments by adding to the restriction digest 5 pmol of *EcoRI* adapters and 50 pmol of *MseI* adapters with 1 unit T4 DNA ligase (Fermentas Inc.) in a total 10 µL buffer comprising 40 mM Tris-HCl (pH 7.8 at 25 °C), 10 mM MgCl₂, 10 mM DTT and 0.5 mM ATP. Ligation was allowed to proceed for 3 h at 37 °C and the mix then diluted twofold with sterile H₂O.

PCR pre-amplification. Two microlitres of the diluted ligation mix was used as the template for the pre-amplification. PCR was performed with 30 ng *Eco-A* and 30 ng of *Mse-C* primers with 0.7 unit DNA polymerase (Expand High Fidelity PCR System, Roche) and 0.2 mM each dATP, dCTP, dGTP, dTTP with 1.5 mM MgCl₂, in a total of 20 µL of the manufacturer's buffer at 1× concentration (composition confidential). The PCR programme consisted of 72 °C for 30 s then 20 cycles of (94 °C for 30 s, 56 °C for 60 s, 72 °C for 60 s). PCR products were diluted fivefold with sterile H₂O.

Selective AFLP amplification. Five *Eco-ANN/Mse-CNN* primer combinations were used for the selective amplification (where N represents any nucleotide): *Eco-AAG/Mse-CAG*, *Eco-ACT/Mse-CTC*, *Eco-ACA/Mse-CAG*, *Eco-ACA/Mse-CAC*, *Eco-AAG/Mse-CTC*.

Six nanograms of *Eco-ANN* primer was radiolabelled with 2 µCi (0.2 µL) of $\gamma^{33}\text{P-ATP}$ (Amersham Biosciences) using 0.25 unit T4 Polynucleotide Kinase (Fermentas), in a total volume of 1 µL buffer comprising 50 mM Tris-HCl (pH 7.6 at 25 °C), 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine and 0.1 mM EDTA. Labelling reactions were performed at 37 °C for 1 h followed by denaturing at 70 °C for 10 min.

Selective amplifications were performed using 3 ng labelled *Eco-ANN* primer and 15 ng *Mse-CNN* primer with 0.35 unit DNA polymerase (Expand High Fidelity PCR System, Roche) and 0.2 mM each dATP, dCTP, dGTP,

dTTP with 1.5 mM MgCl₂, in a total of 10 µL of the manufacturer's buffer. The PCR programme consisted 36 cycles in total: 2 cycles each (94 °C for 30 s, t °C for 30 s, 72 °C for 60 s, where t drops from 65 to 58 in 1 °C steps), followed by 18 cycles of (94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s).

Electrophoresis of radiolabelled PCR products was performed on 6% polyacrylamide gels. These were dried and exposed to X-ray film for 3–5 days at –80 °C. Films were developed following a standard protocol and genotypes scored by hand as a binary matrix of band presence/absence. Each set of 60 reactions included two positive (known genotype) and two negative (H₂O) controls carried from restriction digest through to selective AFLP-PCR.

Data analysis

Dendrochronological data. Trees were assigned to establishment years based on their age. Temperature during establishment year was allocated to each tree from the Turó de l'Home climate record. A 3-year running average of mean annual temperature was used for all climate-establishment analyses. This procedure was based on an assessment of the mean accuracy of tree age measurement of ± 1 year. The 648 m altitudinal range covered by these samples equates to a mean temperature difference of 3.3 °C between the HFL and LFL sites, based on the altitudinal lapse rate of 0.51 °C per 100 m reported by Peñuelas & Boada (2003) for Montseny. Consequently, we adjusted temperature during establishment for each tree according to the mean altitude of each sample site. For initial analysis alongside AFLP data, we created two discontinuous temperature groups by removing samples either side of the median value of 9.06 °C, leaving two samples separated by 1 °C (hot group: 11.47–9.59 °C, cold group: 8.54–6.39 °C). For subsequent marker frequency analyses, age and establishment temperature, data were used to divide trees into sample groups based on age and temperature ranges.

AFLP data. For the estimation of genetic diversity and population differentiation we estimated the frequency of the dominant allele at each locus for each population as $1 - \sqrt{\text{frequency of band absence at that locus}}$ (Krauss 2000). We assumed Hardy–Weinberg equilibrium as previous population genetic work using microsatellite markers found no evidence for deviation from Hardy–Weinberg equilibrium in the studied area of forest (Jump & Peñuelas 2006). At least two alternative approaches have been suggested to reduce potential bias when estimating allele frequencies for dominant markers (Lynch & Milligan 1994; Zhivotovsky 1999). However, Krauss (2000) has shown that biases are largely eliminated in highly polymorphic dominant marker data sets with large numbers of polymorphic loci. In the work reported by Krauss, each of the above procedures for estimating

allele frequencies provided a comparable estimate of heterozygosity when calculated on a data set with 116 AFLP loci and 21 individuals per population — fewer than the number of loci and individuals we used in the current study.

Genetic diversity for each *F. sylvatica* population was calculated as the proportion of polymorphic loci per population, mean heterozygosity and mean Shannon diversity index over all loci using POPGENE, version 1.32 (Yeh & Boyle 1997). To assess population differentiation between samples, Weir and Cockerham's F_{ST} estimator, θ (Weir & Cockerham 1984) was calculated pairwise between the three altitudinal samples and between the hot and cold sample groups, using the software TFPGA (Miller 1997). Population differentiation was tested using an exact test (Raymond & Rousset 1995) performed on marker frequencies using 20 batches, 2000 permutations per batch and 1000 dememorization steps in combination with Fisher's combined probability test using TFPGA. As our LFL sample was not contiguous, we also tested population differentiation and calculated F_{ST} between the two sub groups of the LFL sample. The two LFL sub groups were treated as a single sample as, based on the exact test, they were not significantly differentiated ($F_{ST} = 0.0236$, $\chi^2 = 496_{(508 \text{ d.f.})}$, $P = 0.637$).

Identification of outlier loci (markers potentially under natural selection) was performed using the program DFDIST (© 2005 Beaumont; program distributed by the author), a dominant marker version of the program FDIST described by Beaumont & Nichols (1996). Briefly, allele frequencies are estimated in DFDIST using a method based on that of Zhivotovsky (1999), and F_{ST} (Weir & Cockerham 1984) is calculated for each locus in the sample. Coalescent simulations are then performed to generate data sets with a distribution of F_{ST} close to the empirical distribution. Quantile limits are calculated based on this simulated distribution; loci with unusually low or high values of F_{ST} are regarded as potentially under selection.

DFDIST was used to perform pairwise comparisons of the three altitudinal samples and for comparison of the hot and cold establishment temperature samples. This temperature-based comparison is not independent of the altitude-based sample comparison; however, it controls for interannual variability in temperature that would otherwise cause hot year and cold year trees to be grouped together at each altitude. One hundred thousand (100 000) realizations were performed using the default settings for the program based on a two-deme, two-population model, and a maximum allowable allele frequency of 0.99 pooled across samples. We chose the 0.995 or 0.005 quantiles to define an envelope within which 99% of the data points are expected to lie. Any loci occurring outside these limits were designated as potential outliers.

The significance level of outlying loci was not corrected for multiple tests. Only outlying loci showing a significant

relationship between marker presence/absence and environmental variables were considered to be true outliers. Following the identification of an outlier locus by DFDIST, binary logistic regression was used to predict the probability of marker presence at this locus as a function of altitude, temperature and age. Logistic regression analysis was performed on marker presence/absence data and the value of corresponding predictor variables for each individual using SPSS (version 11.0.2 for Mac, SPSS Inc.). In the results of binary logistic regression we report Nagelkerke r^2 for information only. Hosmer & Lemeshow (2000) note that low r^2 values are the norm in logistic regression, and advise against reporting r^2 owing to difficulties in its interpretation when compared with r^2 from linear regression.

Dominant allele frequency (calculated as above) was assessed at the same outlier locus as a function of altitude, temperature and age. When assessing dominant allele frequency, we compared groups of approximately equal size (N) rather than groups of equal temperature/age range, as random variation in allele frequency increases rapidly with reduction in sample size due to sampling effects. To facilitate comparison between the probability of marker presence predicted from binary logistic regression and dominant allele frequency, probability of the presence of the dominant allele at a locus was predicted from probability of marker presence as $1 - \sqrt{\text{probability of band absence at that locus}}$ (Figs 5 and 6).

Low temperatures limit recruitment at the HFL while high temperatures limit recruitment at the CFA and LFL (A. S. Jump *et al.*, unpublished). To assess temporal trends in marker frequency it was necessary to confine the analysis to the climatically similar CFA and LFL samples. Recruitment at the HFL coincides with periods of warmer temperatures measured at the Turó de l'Home meteorological station; however, the HFL trees still experience colder temperatures than trees at the CFA and LFL, owing to the fall in temperature with increasing altitude. Including the HFL sample in any analysis of temporal trends would cause marker frequency in recent years to be heavily biased by the pulse of recent recruitment in this area (Peñuelas & Boada 2003).

Results

Of the 210 individuals analysed for AFLP, one individual (at the CFA) failed to amplify. The five AFLP primer combinations each produced between 44 and 56 easily scored variable bands between 45 and 550 base pairs across all individuals. Two hundred and fifty-four (254) bands were scored in total. Mean heterozygosity over all samples was 0.242; genetic diversity per sample is given in Table 1. Population differentiation was low, with F_{ST} varying between 0.0134 (CFA vs. LFL) and 0.0181 (HFL vs. LFL) (Table 2). Pairwise population differentiation tests showed

Table 1 Genetic diversity summarized for *Fagus sylvatica* samples in this study

| Population | N | Locus AGTC476 included (254 loci) | | | Locus AGTC476 excluded (253 loci) | | |
|------------|----|-----------------------------------|---------------|---------------|-----------------------------------|---------------|---------------|
| | | % P | H | SI | % P | H | SI |
| HFL | 70 | 89.76 | 0.240 (0.011) | 0.375 (0.014) | 90.12 | 0.241 (0.011) | 0.376 (0.014) |
| CFA | 69 | 92.13 | 0.241 (0.010) | 0.379 (0.014) | 92.09 | 0.240 (0.010) | 0.378 (0.014) |
| LFL | 70 | 95.67 | 0.246 (0.010) | 0.385 (0.014) | 95.65 | 0.245 (0.010) | 0.384 (0.014) |

HFL, high *Fagus* limit (upper treeline); CFA, central forest area; LFL, low *Fagus* limit; N, number of individuals per population; % P, percentage polymorphic loci; H, heterozygosity; SI, Shannon diversity index. H and SI are mean values calculated over loci followed by standard error in parentheses.

Table 2 Population differentiation of *Fagus sylvatica* samples described in this study

| Population comparison | Locus AGTC476 included | | Locus AGTC476 excluded | |
|-----------------------|---------------------------|---|---------------------------|---|
| | F_{ST} | Exact test P | F_{ST} | Exact test P |
| HFL vs. LFL | 0.0181 (0.0120–0.0257) | 0.0008 ($\chi^2 = 615$, 508 d.f.) | 0.0158 (0.0110–0.0211) | 0.0019 ($\chi^2 = 603$, 506 d.f.) |
| HFL vs. CFA | 0.0161 (0.0105–0.0225) | < 0.0001 ($\chi^2 = 675$, 508 d.f.) | 0.0146 (0.0097–0.0202) | < 0.0001 ($\chi^2 = 664$, 506 d.f.) |
| CFA vs. LFL | 0.0134 (0.0077–0.0202) | 0.1723 ($\chi^2 = 538$, 508 d.f.) | 0.0135 (0.0076–0.0206) | 0.1715 ($\chi^2 = 536$, 506 d.f.) |
| Hot vs. cold | 0.0177 (0.0111–0.0264) | < 0.0001 ($\chi^2 = 661$, 508 d.f.) | 0.0146 (0.0099–0.0198) | < 0.0001 ($\chi^2 = 643$, 506 d.f.) |

HFL, high *Fagus* limit (upper treeline); CFA, central forest area; LFL, low *Fagus* limit. Ninety-five per cent (95%) confidence limits of F_{ST} (italicized in parentheses) are based on 10 000 bootstrap resamples over loci. Values of χ^2 are given in parentheses in normal type in exact test columns. Significantly differentiated samples are shown in bold type.

significant differentiation between the HFL and CFA samples and between the HFL and LFL samples ($P < 0.05$), whereas the CFA and LFL samples were not significantly differentiated ($P > 0.05$, Table 2).

Outlier analysis

When the HFL and LFL samples were compared, outlier tests performed using *DFDIST* detected a single outlier locus with unusually high F_{ST} . This outlier, locus AGTC476 ($F_{ST} = 0.301$, $P = 0.004$; Fig. 3a), was clearly separate from the distribution of other loci. The same locus was situated just within the 0.995 quantile when the HFL and CFA samples are compared ($F_{ST} = 0.217$, $P = 0.018$, Fig. 3b). When the CFA and LFL samples were compared, locus AGTC476 was situated close to the median simulated F_{ST} at this heterozygosity ($F_{ST} = -0.0002$, $P = 0.501$; Fig. 3c). No outlying loci were detected in the CFA vs. HFL and CFA vs. LFL comparisons.

When samples were ordered by establishment temperature and the outlier analysis was repeated between hot and cold year trees, locus AGTC476 was again detected as an outlier. In this comparison, F_{ST} for this locus was the

highest detected in any of the pairwise comparisons conducted ($F_{ST} = 0.361$, $P = 0.002$; Fig. 4).

Locus AGTC476 marker frequency analyses

The frequency of the dominant allele at locus AGTC476 (allele AGTC476d) decreases with decreasing altitude, from fixation (1.0) at the HFL (1640 m a.s.l.) to 0.681 at the CFA (1127 m a.s.l.) and 0.604 at the LFL (992 m a.s.l.). The frequency of this allele also falls with increasing temperature during establishment (Fig. 5). Probability of marker presence at locus AGTC476 is significantly related to altitude [logit $P(y) = -4.96 + 0.006x$, Nagelkerke $r^2 = 0.184$, $\chi^2 = 15.49$, d.f. = 1, $P < 0.001$]. However, the relationship with altitude-standardized temperature is stronger [logit $P(y) = 12.98 - 1.11x$, Nagelkerke $r^2 = 0.231$, $\chi^2 = 19.74$, d.f. = 1, $P < 0.001$] than with altitude alone. There is no relationship between marker frequency and establishment year over all three sample sites ($\chi^2 = 0.15$, d.f. = 1, $P = 0.70$). Samples with mean annual temperature during establishment at or below 8.1 °C were fixed for the presence of allele AGTC476d whereas its frequency fell to 0.47 for the sample of trees established during the warmest

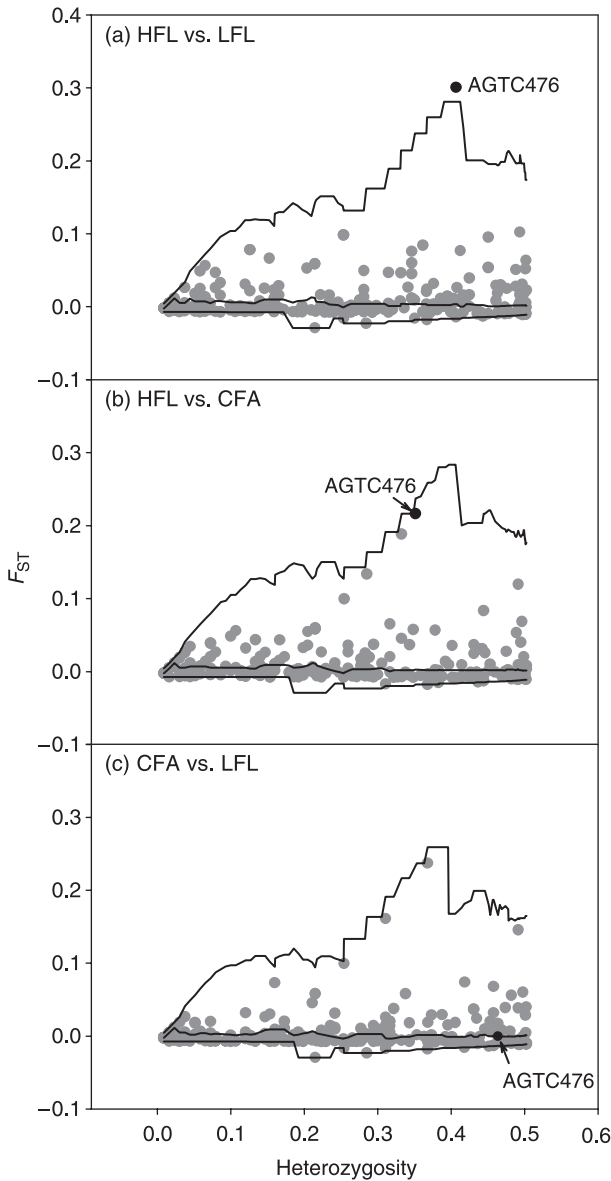


Fig. 3 Pairwise comparison of the distribution of per-locus F_{ST} of *Fagus sylvatica* samples from three different altitudes, HFL: upper treeline (high *Fagus* limit), $N = 70$; CFA: central forest area, $N = 69$; LFL: low *Fagus* limit, $N = 70$. Estimated values of F_{ST} from 254 AFLP loci are plotted as a function of heterozygosity. Solid lines denote 0.995, 0.5 (median) and 0.005 quantiles of the conditional distribution obtained from coalescent simulations. The outlying locus AGTC476 detected in the HFL vs. LFL comparison is highlighted.

years (Fig. 5). Allele frequency decreases with increasing temperature by an average of 0.135 per °C when calculated over the six temperature groups of the frequency analysis shown in Fig. 5.

If the HFL samples are excluded (thereby analysing only the climatically similar CFA and LFL samples), the probability of marker presence at locus AGTC476 is not significantly

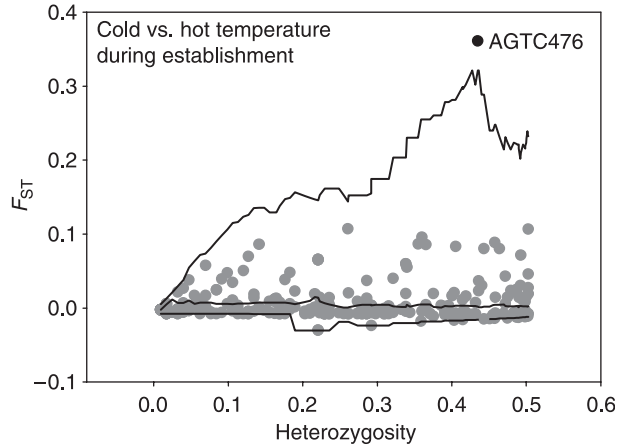


Fig. 4 Pairwise comparison of the distribution of per-locus F_{ST} of *Fagus sylvatica* samples from cold and hot mean annual temperature during establishment. Cold sample group: 6.39–8.54 °C, 97% HFL, 3% CFA trees, $N = 68$. Hot group: 9.59–11.47 °C, 25% CFA, 75% LFL trees, $N = 64$. Solid lines denote 0.995, 0.5 (median) and 0.005 quantiles as Fig. 3. The outlying locus AGTC476 is highlighted.

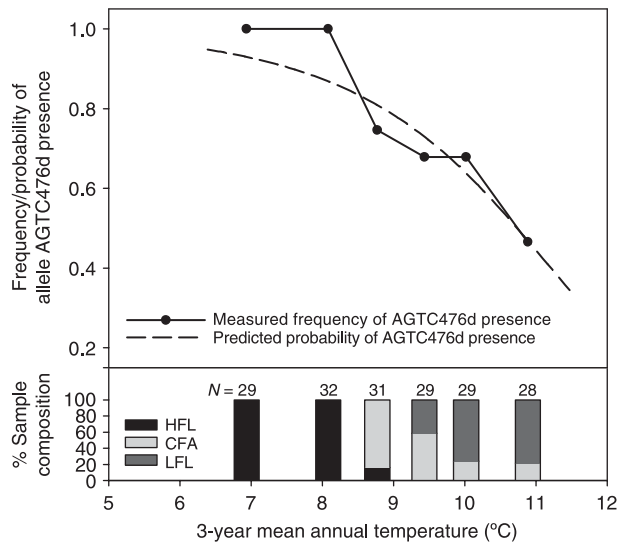


Fig. 5 Upper panel: Frequency of allele AGTC476d as a function of temperature during *Fagus sylvatica* establishment and probability of the presence of this allele. Probability of AGTC476d presence is calculated as 1 – the square root the probability of marker absence at locus AGTC476 predicted from logistic regression of marker presence/absence data on establishment temperature [logit $P(y) = 12.98 - 1.11x$, $\chi^2 = 19.74$, d.f. = 1, $P < 0.001$]. Temperature recorded at Turó de l’Home meteorological station (1712 m above sea level) was adjusted by sample altitude, based on the lapse rate of 0.51 °C per 100 m reported by Peñuelas & Boada (2003). Lower panel: Sample sizes and composition of samples for the temperature-based marker frequency analysis. HFL, upper treeline (high *Fagus* limit); CFA, central forest area; LFL, low *Fagus* limit.

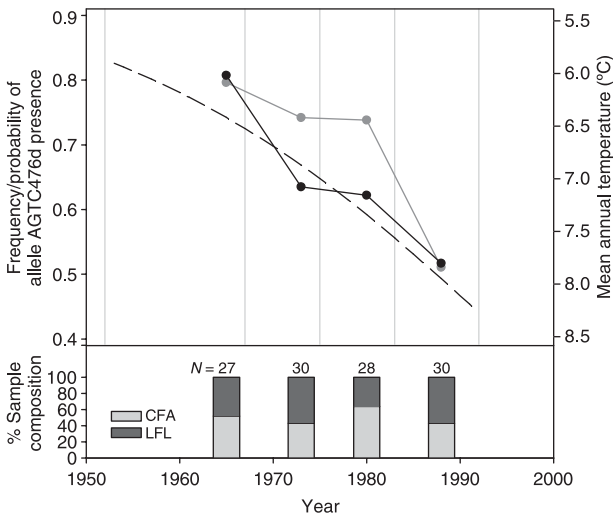


Fig. 6 Temporal variation in the frequency of allele AGTC476d and probability of the presence of this allele within the CFA and LFL samples. Probability of AGTC476d presence is calculated as $1 - \sqrt{\text{probability of marker absence at locus AGTC476d}}$ predicted from logistic regression of marker presence/absence data on establishment year [logit $P(y) = 137.59 - 0.69x$, $\chi^2 = 5.63$, d.f. = 1, $P = 0.018$]. Allele frequency is plotted against the modal year in each sample group with vertical grey lines representing the range of years in each group. Mean annual temperature recorded at Turó de l'Home meteorological station during the modal sample year is plotted on an inverted axis to facilitate comparison between the plots. Lower panel: Sample sizes and composition of samples for the time-based marker frequency analysis. CFA, central forest area; LFL, low *Fagus* limit —●— Temperature; —●— Measured frequency of AGTC476d presence; --- Predicted probability of AGTC476d presence.

related to altitude ($\chi^2 = 1.18$, d.f. = 1, $P = 0.28$). However, the negative relationship between establishment temperature and probability of marker presence persists [logit $P(y) = 10.33 - 0.85x$, Nagelkerke $r^2 = 0.098$, $\chi^2 = 6.29$, d.f. = 1, $P = 0.012$]. Probability of marker presence at the CFA and LFL sites also shows a declining trend with decreasing tree age [logit $P(y) = 137.59 - 0.69x$, Nagelkerke $r^2 = 0.088$, $\chi^2 = 5.63$, d.f. = 1, $P = 0.018$]. To reflect the dominance of particular years in the establishment record, in Fig. 6 allele frequency data are presented as a function of the modal sample year for each age group. By the most recent analysis period (1983–1992), frequency of allele AGTC476d had fallen by 36% compared to its initial 1952–1967 value. This decline tracks the increase in mean annual temperature over the same period.

Discussion

Given the large population from which our samples are drawn (c. 2830 ha near-continuous forest) and that *Fagus sylvatica* is a highly outcrossing, wind-pollinated species, average levels of linkage disequilibrium (LD) across the

genome of this species are expected to be low. In *Populus tremula* (European aspen), which is also outcrossing and wind pollinated, Ingvarsson (2005) found that average levels of LD are generally low, declining to negligible levels in less than 500 bp in five nuclear genes studied at the species level. At the population level, LD was generally two to five times higher in the same genes, extending beyond 1–2 kb. Linkage disequilibrium may be substantially higher around loci that have experienced recent directional selection (Ingvarsson 2005). The genomes of *F. sylvatica* (544 Mb, Gallois *et al.* 1999) and *Populus* (550 Mb, Taylor 2002) are small relative to other trees. However, the 254 loci used in our study give an average of only one marker per 2.14 Mb of the genome of *F. sylvatica* (although we have no information on the distribution or potential aggregation of loci across the genome). The likelihood of finding a marker linked to an adaptively important gene in *F. sylvatica* in our study is therefore relatively low if LD is comparably low in both taxa.

Adaptive differentiation at high and low altitude limits

Despite the low likelihood of finding a marker linked to an adaptively important gene, the results that we report here show that trees at the upper and lower altitudinal limits of *F. sylvatica* are exceptionally differentiated at the AGTC476 locus (Fig. 3a) when compared with other regions of the genome. Differentiation at this locus is significantly greater than that expected under selective neutrality; therefore this locus is a candidate for marking a region of the *F. sylvatica* genome subject to natural selection operating between the upper and lower limits of this species' distribution.

Dominant allele frequency at locus AGTC476 increased from 0.604 at the species' lower limit to fixation (1.0) at the upper treeline. The most marked environmental gradient over the same distance is one of temperature, which differs by 3.3 °C between the upper and lower sample sites based on their altitude. Climatic adaptation is a highly important component in the evolution of temperate tree species with temperature potentially the most important selective agent over an altitudinal cline (Saxe *et al.* 2001). Dendroecological work shows that this temperature difference has a major ecological impact on the trees in these sites, with greater growth and establishment being correlated with warm temperatures at the HFL and with cool temperatures at lower altitudes (A. S. Jump *et al.*, unpublished).

The differentiation at locus AGTC476 between the cold and hot temperature groups ($F_{ST} = 0.361$; Fig. 4) was even greater than when the upper and lower limits of the species were compared ($F_{ST} = 0.301$; Fig. 3a). Although the cold year sample was made up almost exclusively of HFL trees, the hot year sample consisted of a mixture of trees established at lower altitudes. These CFA and LFL samples were undifferentiated at AGTC476 and over all loci

(Fig. 3c, Table 2). Whereas the evidence for selection at AGTC476 between the HFL and CFA was not convincing (Fig. 3b), the combining of HFL and CFA trees in the temperature-based comparison increased rather than decreased differentiation at this locus. When viewed together with the known importance of temperature as a selective agent over the studied cline, this strongly suggests that temperature is imposing the selective pressure detected at this locus. Excluding AGTC476 reduced the overall differentiation between all sample comparisons except CFA vs. LFL, where there was clear evidence that this locus is not under selection (Table 2, Fig. 3c). The exclusion of this locus has little effect on overall estimates of genetic diversity (Table 1).

Marker frequency linked to spatio-temporal temperature variation

If the frequency of allele AGTC476d is linked to temperature, then it should vary predictably as establishment temperature increases across multiple sample groups, irrespective of the altitudinal origin of their constituent trees. Figure 5 shows this to be the case. Probability of AGTC476d presence declines with increasing temperature, with trees establishing during lower temperatures being more likely to possess this allele. As establishment temperature rises, so the frequency of this allele falls. This relationship is demonstrated by the significant logistic regression of marker presence/absence on temperature data ($P < 0.001$). Altitude is not a significant predictor of probability of marker presence if HFL individuals are excluded ($P = 0.28$). Critically, probability of marker presence is still significantly related to temperature when the effects of altitude and population differentiation are controlled by the removal of HFL individuals from the logistic regression analysis ($P = 0.012$). The decline of AGTC476d frequency over time in parallel with increasing mean annual temperature is evident in Fig. 6. The pattern of decreasing frequency of this allele with increasing temperature parallels the altitudinal cline reported above, thereby providing additional correlative support for temperature as the selective agent acting on this locus.

A similar pattern of genetic differentiation with respect to high and low establishment temperature is reported in *Betula pendula* (European white birch) by Kelly *et al.* (2003). They used a correlative approach based on principal coordinates analysis to assess subpopulation differentiation according to temperature in establishment year. Kelly *et al.* report significant interannual genetic differentiation of 'warm year' and 'cool year' multilocus genotypes of 13 trees establishing in a 2-ha forest plot. The temperature-linked population differentiation we report is confined to locus AGTC476. Unlike Kelly *et al.* (2003) we do not find a separation of groups of multilocus genotypes according to

establishment temperature using principal coordinates analysis (data not shown). It would be interesting to see if the action of natural selection could be inferred in causing the observed pattern in birch and candidate loci identified if the study of Kelly *et al.* were repeated with a larger sample size. Introgression from a related species can also create locus-specific deviations from neutrality (Lexer *et al.* 2004). Hybridization is known to occur between *Betula pendula*, *Betula pubescens* and *Betula nana* (Palme *et al.* 2004); however, although *F. sylvatica* hybridizes with *Fagus orientalis*, these species do not co-occur in the region we studied (Comps *et al.* 2001).

Evolutionary response to climate change

Temperatures recorded at Turó de l'Home meteorological station show a strong warming trend. By 2003, temperatures had increased by approximately 1.65 °C when compared with the 1952–1975 mean (Fig. 2). This rapid rise in temperature is likely to represent a strong selection pressure (Davis & Shaw 2001; Davis *et al.* 2005; Jump & Peñuelas 2005; Thomas 2005). In the studied population of *F. sylvatica*, the frequency of allele AGTC476d shows a predictable response to interannual variation in temperature (Fig. 5). We therefore hypothesized that recent climatic warming would have imposed directional selection pressure at this locus, leading to decreasing frequency of AGTC476d in successive cohorts of trees established from the seed pool in recent years. A significant decrease in the probability of marker presence at locus AGTC476 does occur ($P = 0.018$). This decrease is evident in Fig. 6, which shows the declining frequency and probability of AGTC476d presence at the CFA and HFL over time in parallel with rising temperatures in the region over the last half-century.

In common with the correlative studies described above and those reviewed by Jump & Peñuelas (2005), this work demonstrates that adaptive climatic differentiation occurs between individuals within populations, not just between populations throughout a species geographic range (Davis & Shaw 2001). We hypothesize that this temperature-linked polymorphism is maintained within the population by balancing selection resulting from interannual variability in climate. An effect of this polymorphism is that some genotypes in a population may be 'pre-adapted' to warmer temperatures (Davis & Shaw 2001). The increase in frequency of these genotypes, which has occurred in our study in parallel with rising temperatures, shows that current climatic changes are now imposing directional selection pressure on the population. The change in allele frequency that has occurred in response to this selection pressure also demonstrates that a significant evolutionary response can occur on the same timescale as current changes in climate (Davis *et al.* 2005; Jump & Peñuelas 2005; Thomas 2005).

Jump & Peñuelas (2005) suggested that temperature-related genetic variability within populations should increase the population's ability to tolerate changes in climate. Our data show that, at least in *F. sylvatica*, limited tolerance of climate change may be possible through evolutionary changes based on pre-existing genetic variability within the population. This demonstrates that evolution is inherent in the persistence of populations under a changing climate, as Davis *et al.* (2005) argue. However, in agreement with the work of Kelly *et al.* (2003) we find that, in the short term, an evolutionary response to climate change is not necessarily dependent on the supply of new variation from populations in other areas of the species' range. If as our data suggest, the AGTC476 locus is linked to a region of the *F. sylvatica* genome under selection by temperature, differential temperature sensitivity of individuals possessing or lacking the AGTC476d allele would enable them to coexist through temporal fluctuation in their recruitment from the seed pool linked to normal interannual climatic variability (Kelly & Bowler 2005). However, climatic warming would confer a selective advantage on individuals pre-adapted to warmer temperatures (here those lacking AGTC476d), leading to the proportionate increase of these individuals within the population that we report.

In the short term, the population we studied may adapt to climatic warming by an increase in the proportion of individuals that are pre-adapted to warmer temperatures. However, as the climate continues to warm, gene flow from populations in warmer regions of the species range may be necessary to allow these populations to continue to adapt (Billington & Pelham 1991; Davis & Shaw 2001; Jump & Peñuelas 2005). The position of this population at the southern edge of the species' latitudinal range means that supply of useful new genetic variation from populations already adapted to warmer temperatures is not likely (Thomas 2005). It is therefore unlikely that low altitude populations of *F. sylvatica* in this region will be able to adapt to rising temperatures in the long term (Davis & Shaw 2001; Davis *et al.* 2005; Jump & Peñuelas 2005; Thomas 2005). Over the last half-century, *F. sylvatica* has shown a migration to higher altitudes in the Montseny region and the gradual extinction of isolated populations at its lower range-edge, linked to rising temperatures and land use change (Peñuelas & Boada 2003). Thus, although we present evidence for a recent evolutionary response to rising temperatures, it is not enough to enable this species to persist in all of its current locations. Predicting the long-term effects of directional climate change on the population will be aided by the characterization of the phenotypic differences between subpopulations possessing and lacking the AGTC476d allele.

The findings from our work across the altitudinal range of this species can not easily be generalized to its latitudinal

range, as it is unlikely that gene flow is high enough to connect populations of *F. sylvatica* across Europe. A priority for future work on *F. sylvatica* should be to assess how latitudinal patterns in the frequency of this temperature polymorphism mirror those of altitude described here. Efforts should be made to determine what phenotypic differences exist between those individuals possessing and lacking the AGTC476d allele and to identify the genes that underlie this difference. Additionally, it is important to determine whether this pattern of adaptive genetic differentiation can be generated experimentally in other species. For this purpose, ongoing long-term climatic manipulation experiments represent a unique resource.

Conclusions

Our study shows that temperature-related adaptive differentiation occurs between individuals at the upper and lower altitudinal limits of *Fagus sylvatica*. We hypothesize that this temperature-linked polymorphism is maintained at lower altitudes by balancing selection resulting from normal interannual variability in climate. Recent increases in temperature as a result of global climate change are imposing a directional selection pressure on the population, resulting in the directional genetic change that we report here. In the studied population, an evolutionary response to warming temperatures is underway. However, although we show that this population has some capacity for short-term adaptation, this is not enough to allow this species to persist in all of its current locations as ongoing changes in the species' distribution demonstrate.

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