Disentangling the Roles of History and Local Selection in Shaping Clinal Variation of Allele Frequencies and Gene Expression in Norway Spruce (*Picea abies*)

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ABSTRACT Understanding the genetic basis of local adaptation is challenging due to the subtle balance among conflicting evolutionary forces that are involved in its establishment and maintenance. One system with which to tease apart these difficulties is clines in adaptive characters. Here we analyzed genetic and phenotypic variation in bud set, a highly heritable and adaptive trait, among 18 populations of Norway spruce (*Picea abies*), arrayed along a latitudinal gradient ranging from 47°N to 68°N. We confirmed that variation in bud set is strongly clinal, using a subset of five populations. Genotypes for 137 single-nucleotide polymorphisms (SNPs) chosen from 18 candidate genes putatively affecting bud set and 308 control SNPs chosen from 264 random genes were analyzed for patterns of genetic structure and correlation to environment. Population genetic structure was low ($F_{ST} = 0.05$), but latitudinal patterns were apparent among Scandinavian populations. Hence, part of the observed clinal variation should be attributable to population demography. Conditional on patterns of genetic structure, there was enrichment of SNPs within candidate genes for correlations with latitude. Twenty-nine SNPs were also outliers with respect to F_{ST} . The enrichment for clinal variation at SNPs within candidate genes (i.e., SNPs in *PaGI*, *PaPhyP*, *PaPhyN*, *PaPRR7*, and *PaFTL2*) indicated that local selection in the 18 populations, and/or selection in the ancestral populations from which they were recently derived, shaped the observed cline. Validation of these genes using expression studies also revealed that *PaFTL2* expression is significantly associated with latitude, thereby confirming the central role played by this gene in the control of phenology in plants.

LOCAL adaptation is a key process in the evolution of species. Understanding how local adaptation is established and maintained, however, is especially difficult as its establishment is contingent upon historical conditions and its maintenance depends on the balance among conflicting evolutionary forces (e.g., Yeaman and Otto 2011). It is

a particularly challenging task in forest trees, because they have long generation times and therefore cannot be easily manipulated experimentally. For instance, transfer experiments are theoretically possible but practically difficult to implement. On the other hand, the analysis of the strong latitudinal clines displayed by forest trees for potentially adaptive traits such as bud set (Dormling 1973; Savolainen *et al.* 2007; Aitken *et al.* 2008) can provide crucial information on the forces involved in local adaptation and, in particular, on the relative parts played by demography and selection in the establishment of the cline. Furthermore, phenology in general, and flowering time and bud set in particular, have been extensively studied and strong candidate

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genes are available, many of which belong to the photoperiodic pathway including the circadian clock (Gyllenstrand *et al.* 2007; Albani and Coupland 2010; Bergelson and Roux 2010; Fornara *et al.* 2010; Holliday *et al.* 2010; Hsu *et al.* 2011). Recent candidate gene studies on the genetic basis of clinal variation in phenology in European aspen (Ma *et al.* 2010) and Sitka spruce (Holliday *et al.* 2010; Lobo 2011) illustrate well the promises of this approach.

In the present study we focus on a conifer species, Norway spruce [*Picea abies* (L.) Kar.] and more specifically on clinal variation in the northwest part of its natural range. The clines in phenological traits observed in this species and in this part of its range are intriguing for several reasons. First, as in many other forest tree species (Jaramillo-Correa *et al.* 2001; Savolainen *et al.* 2007; Alberto *et al.* 2011; Kremer *et al.* 2010; Prunier *et al.* 2011), Norway spruce populations are strongly differentiated for bud set [$Q_{ST} = 0.74$ (R. Liesch, unpublished data)], a trait with high heritability [average $h^2 = 0.63$ (varying from 0.33 to 0.78 across 15 populations) (R. Liesch, unpublished data)]. This strong level of phenotypic differentiation for bud set contrasts with extensive gene flow and limited genetic differentiation among populations at neutral genetic markers. Genetic structure within and among Norway spruce populations is largely accounted for by two major geographically based groups ($F_{ST} \sim 0.10$ between groups; $F_{ST} \sim 0.05$ within groups), the Nordic-Baltic group and the Alpine group (Heuertz *et al.* 2006). Second, tree populations in Northwestern Europe are young, as most of the region was covered by ice during the Last Glacial Maximum (LGM) ($\sim 18,000$ YBP). The combined use of palynological data, macrofossils, and maps of genetic diversity suggests that Norway spruce recolonized Scandinavia after the LGM from populations located in central Russia and possibly also from cryptic refugia in the northern part of European Russia (Binney *et al.* 2009; Välimäki *et al.* 2011). The spread westward followed two main recolonization routes, a northern route over Finland and into northern Scandinavia and a southern route going through the Baltic states (Tollefsrud *et al.* 2009). Norway spruce reached eastern Finland ~ 6500 years ago and east-central Sweden ~ 2700 years ago, after which it took 100–500 years to reach the present population size and to replace the existing *Pinus–Betula–Alnus* forests (Giesecke and Bennett 2004; Seppä *et al.* 2009). This history is reflected in a complex population genetic structure across the extant range of this species despite extensive gene flow (Heuertz *et al.* 2006).

The recolonization of Scandinavia from different LGM refugia is not specific to Norway spruce and has been observed in various plant and animal species (*e.g.*, Jaarola *et al.* 1999; De Carvalho *et al.* 2010). Because Scandinavia was recolonized very recently and from different refugia, the presence of steep clines in phenological traits raises important questions. Do the clines reflect local adaptation on *de novo* mutations occurring only after the recolonization, *i.e.*, under strong local selection over a very short period of

time? Or do they reflect the deployment in space of polymorphisms already segregating in the ancestral populations, *i.e.*, through soft sweeps (Pritchard *et al.* 2010; Savolainen *et al.* 2011)? A correct interpretation of the clines, thus, requires that population history and the ensuing population genetic structure (*e.g.*, pattern of isolation-by-distance) are taken into account. This is because differences in allele frequencies among populations (*e.g.*, between southern and northern Swedish spruce populations) could be due to expansion from different glacial refugia where selection, or simply random genetic drift, led to the fixation of different alleles. This, of course, would not necessarily imply that local selection favoring different alleles in different areas does not occur, but it would nonetheless imply that it might not be the only explanation for the observed clinal variation. Finally, clinal variation in phenology could also partly reflect phenotypic plasticity, an aspect that was not investigated here, but that is important in responses by tree populations to climate (see Bresson *et al.* 2011).

In the present study, after validating the presence of clinal variation in bud set in a subsample of five populations, we examined single-nucleotide polymorphism (SNP) frequencies at candidate genes for timing of bud set in 18 Norway spruce populations for correlations with latitude (Supporting Information, Figure S1). Various approaches, capturing different facets of the data, were used to detect and interpret the clinal variation at candidate gene SNPs. We then tested for clinal variation in gene expression in a subset of the genes for which clinal variation was detected for SNP allele frequencies. This study offers a clear illustration of the interplay between demographic history and selection in shaping genetic variation and of the intrinsic difficulty in telling the two apart (Li *et al.* 2012).

Materials and Methods

Sampling and measurement of bud set

Seeds of 303 Norway spruce trees were collected from 18 populations in four countries: Germany (1), Russia (1), Finland (7), and Sweden (9) at latitudes ranging from 47°N to 68°N (see Table 1 and Figure 1). To ascertain the presence of clinal variation in bud set, seeds were randomly sampled from 5 populations [SE-58.1 (Saleby), SE-61.6 (Istevallen), SE-62.7 (Högmansbod), SE-64.1 (Jämtland), and SE-66.4 (Jock)] with latitudes ranging from 58°N to 66°N. Populations from northern Finland, which were used to analyze clinal variation in allele frequency, were not used here and were replaced by population SE-66.4 since their seeds had low germination rates. The number of seeds per population varied between 36 and 48 and the number of maternal trees from 13 to 20. The seeds were germinated and the seedlings were then cultured in a growth chamber under continuous light ($250 \mu\text{mol m}^{-2} \text{sec}^{-1}$ light and 400–700 nm) and a temperature of 20° for 8 weeks. Thereafter the seedlings were grown under increasing night length. Each photoperiod lasted 1 week starting with 1 week of

Table 1 Populations used in this study

Group	Population code	Population name	Country	No. individuals	Latitude (°N)	Longitude (°E)	Altitude (m)
Alpine domain	GE-47.0	Ruhpolding	Germany	30	47.00	12.23	1492
	SE-58.3	Saleby	Sweden	15	58.37	13.15	70
Baltic–Nordic domain							
Russia	RU-53.3	Bryansk	Russia	15	53.30	34.30	179
Central Fennoscandia	FI-61.5	StKu19	Finland	19	61.57	29.22	105
	SE-61.6	Istevallen	Sweden	13	61.62	16.48	350
	SE-61.8	Tjarnelund	Sweden	16	61.83	16.28	285
	FI-62.0	StKu7	Finland	16	62.07	24.48	105
	SE-62.6	Strangsund	Sweden	21	62.63	15.05	415
	SE-62.7	Hogmansbod	Sweden	15	62.78	15.23	350
	FI-63.0	StKu5	Finland	15	63.07	30.28	170
	SE-63.4	Mittberget	Sweden	14	63.48	17.67	300
	SE-63.7	Hallen	Sweden	13	63.74	15.50	375
	SE-64.1	Jämtland	Sweden	17	64.17	19.36	355
	SE-65.3	Lillpite	Sweden	18	65.31	18.70	242
	SE-66.4 ^a	Jock	Sweden	48	66.41	22.44	145
	FI-66.4	StKu2	Finland	12	66.40	26.88	175
	FI-67.0	Kolari	Finland	27	67.04	26.36	264
	FI-67.7	StKu19	Finland	17	67.72	26.05	280
	FI-68.0	Muonio	Finland	10	68.00	24.15	342

^aSE-66.4 was used only in the expression study.

continuous light (LL), then 1 week with 22 hr light/2 hr dark, and so on. The dark period was extended by 1.5 hr each week until a photoperiod of 14.5 hr light/9.5 hr dark was reached. The latter lasted for 2 weeks (see Figure S2 for a detailed schedule).

Bud set was defined as two categories: 0, no bud at all; and 1, when the bud started to appear until bud set was complete. Bud set was measured at the end of every photoperiodic treatment from week 5 (17.5 hr light/6.5 hr dark) to week 8 (14.5 hr light/9.5 hr dark). The number of individuals that had set bud was counted in each population.

DNA extraction and SNP genotyping

Genomic DNA was extracted from megagametophytes using the DNeasy Plant Mini Kit (QIAGEN, Germantown, MD) and then amplified using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences, Piscataway, NJ). A total of 445 SNPs representing 282 genes were genotyped using an Illumina GoldenGate 768 SNP array. The SNPs used in this study come from three different sources. The first set of 142 SNPs, including 137 candidate SNPs, comes from our own resequencing efforts in a discovery panel of 48 Norway spruce alleles and comprises mostly SNPs from 18 putative candidate genes for bud set. The candidate genes were chosen among genes of the photoperiodic control pathway in *Arabidopsis thaliana* (Fornara *et al.* 2010) and based on information provided by experiments on bud set (*e.g.*, Gyllenstrand *et al.* 2007) or on specific gene families (Karlgrén *et al.* 2011) in Norway spruce. They were putatively involved in the photoperiodic pathway [PaCOL1, PaCOL2, PaCRY, PaPHYN, PaPHYO, PaPHYP, and PaFTL2 and its promoter (formerly called FT4 in Gyllenstrand *et al.* 2007)], the circadian clock (PaCCA1 and its promoter,

PaPRR1, PaPRR3, PaPRR7, and PaGI), or shoot apical development (PaMFTL1, PaHB3, PaKN1, PaKN2, and PaKN4) of Norway spruce (see Table S1 for details). Multiple haplotypes were sequenced for each gene with an average number of 42 haplotypes per gene, except for three PaGI fragments for which only 8 haplotypes were sequenced. The second source of 83 SNPs is the Comparative Re-Sequencing in Pinaceae (CRSP) project (<http://dendrome.ucdavis.edu/crsp/>). Sequences used for SNP discovery came from 800 primer pairs originally designed for loblolly pine (*Pinus taeda* L.). This list of primer sets was constructed without knowledge of putative gene function and was based solely on the set of 800 that maximized the production of high-quality sequence in single validation samples from each of the seven species composing the CRSP project. A single tree from Germany was used as the validation sample for Norway spruce. The SNP discovery panel consisted of 12 megagametophytes sampled in Poland, Latvia, Romania, Belarus, Ukraine, Germany, and Norway, which are fairly representative of the whole range. Finally, the remaining 220 SNPs come from the Arborea project (<http://www.arborea.ulaval.ca/>) as follows. A total of 1964 SNPs from 1485 genes initially identified and successfully genotyped in *P. glauca* were tested in 12 (diploid) individuals of Norway spruce from 12 populations in central Europe, including Latvia, Poland, and Germany. These 1485 genes were transcription factors or candidate genes related to growth, wood formation, and cell wall and lignin synthesis, which were deduced from expression studies in *P. glauca* or annotations in *Arabidopsis* homologs. Testing was done by submitting the panel directly to two Illumina GoldenGate SNP genotyping chips (Beaulieu *et al.* 2011; Pelgas *et al.* 2011), resulting in the discovery of 384 valid SNPs from 340 genes (transfer

Figure 1 Geographic range of Norway spruce (*Picea abies*) and location of the population samples used in this study. The area shaded in green is the distribution of Norway spruce (modified from EUFORGEN 2009, www.euforgen.org) and the dots are the geographical locations of the populations used in this study.

rate of 20%). Alleles with a frequency $<10\%$ were removed from all SNP discovery panels. In summary, SNPs that were genotyped successfully were grouped into two categories: 137 candidate SNPs originating from 18 genes putatively involved in the photoperiodic pathway and 308 control SNPs originating from 264 genes not related to these functions. Importantly, the available information on the involvement of these candidate genes in the control of bud set is variable and consists of information in model species (*A. thaliana* and poplars) and physiological studies in Norway spruce. For example, while the level of expression of *PaFTL2* correlates with the timing of bud set in Norway spruce (Gyllenstrand *et al.* 2007), no such direct evidence exists for other candidate genes. It is also worth noting that since many of the statistical tests used in this study are based on testing whether the number of candidate gene SNPs is overrepresented among the statistically significant SNPs, enlarging the candidate gene category with less well-characterized genes will make these tests more conservative as enrichment will be less likely.

Linkage disequilibrium

Clinal variation of allele frequencies at SNPs unrelated to the targets of natural selection can be generated through linkage. Linkage disequilibrium (LD) among all pairs of

SNPs, therefore, was estimated using the program Arlequin ver. 3.5 (Excoffier and Lischer 2010). Significant LD was identified at the 5% level using chi-square tests with a Bonferroni correction for multiple comparisons and was based on r^2 (Hill and Robertson 1966). Analysis was conducted for each population separately.

Genetic structure

To investigate population genetic structure, we used the Bayesian algorithm that is implemented in the program STRUCTURE ver. 2.3.3 (Pritchard *et al.* 2000; Hubisz *et al.* 2009) to delineate clusters of individuals on the basis of their multilocus genotypes. All 18 populations and 264 unlinked control loci were used in this analysis. Linkage disequilibrium among SNPs within genes was limited as the number of SNPs per gene was small (see below). An admixture model with correlated allele frequencies was chosen and the analysis was performed for a number of clusters varying from $K = 1$ to $K = 22$. For each value of K we performed 20 independent runs. Each run comprised 10,000 iterations of burn-in and 10,000 MCMC steps, as those values were found to be sufficient in pilot runs [longer burn-in (100,000) and MCMC (1,000,000) did not significantly change the results]. To determine the optimal K value, we used both the mode of posterior

probabilities $\text{LnPr}(X | K)$ (Pritchard *et al.* 2000) and ΔK (Evanno *et al.* 2005).

Mantel tests were performed to test for isolation-by-distance among all sampled populations. A Mantel test of the correlation between a matrix of pairwise F_{ST} between populations and the matrix of geographic distances was performed using the *vegan* library in R (Oksanen *et al.* 2011; R Development Core Team 2011). The ratio ($F_{ST}/(1 - F_{ST})$) and the logarithm of geographic distance were used in this test as suggested by Rousset (1997). Finally, we calculated hierarchical F -statistics using the *hierfstat* library in R (Goudet 2005).

Analysis of clinal variation in allele frequencies

To detect candidate genes potentially involved with local adaptation, we treated each SNP independently and used different statistics to estimate the association between population allele frequencies and latitude. For all statistics, we examined the enrichment of SNPs located in candidate genes by calculating the ratio of candidate to control SNPs among those that were significant at 1% or 5% for the test statistics under consideration. We then used bootstrap resampling to assess the significance of the observed excess. Using this method should minimize the number of false positives, contingent upon the control SNPs largely reflecting neutral processes. By comparing the results obtained with the different methods, we obtained a set of candidate SNPs with a geographic pattern clearly distinct from the pattern at control SNPs.

Linear regression on latitude: If geographic variation is associated to an environmental gradient covarying with latitude, we expect the allele frequencies to be clinal along latitudinal gradients. Ancestral and derived alleles at each SNP were determined using the southernmost population (RU-53.3, Bryansk) and nine individuals randomly sampled from four Brewer spruce (*P. breweriana* S. Watson) populations as outgroups. In the few cases of conflict, we chose the SNP in Brewer spruce as the ancestral state. To measure the association of allele frequencies with population latitude, allele frequencies were first transformed using a square-root arcsine function (Berry and Kreitman 1993) and then regressed on population latitude. On the basis of the results of the population structure analysis, three different data sets were considered when analyzing clines. We first considered the complete data set. We then excluded the three southernmost populations. The GE-47.0 (Ruhpolding) and SE-58.3 (Saleby) populations belonged to a different cluster than other populations (see below). These two populations will create a pattern similar to the latitudinal cline, and we therefore excluded them from this analysis. The RU-53.3 (Bryansk) population is located much farther south from the rest of the populations and to avoid possible false positives, we excluded it. Finally, we excluded the four northernmost populations (FI-66.4, FI-67.0, FI-67.7, and FI-68.0)

and considered a set including only populations from central Fennoscandia. Since the second data set seems the most appropriate for the analysis of clinal variation, it was analyzed in more detail than the two others.

Since the coefficient of determination, R^2 , is a measure of the proportion of the total variance in frequencies that is explained by latitude, we used it as a statistic for “clinality” (Berry and Kreitman 1993). The significance of the R^2 value for each candidate SNP was obtained empirically by comparison to the distribution of R^2 values from the control SNPs and enrichment of significant SNPs among the candidate SNPs was tested for as described previously.

Regression Monte Carlo simulation: To investigate to what extent a significant clinal variation observed at one nucleotide site could be due to LD with another SNP, we used the Monte Carlo simulation approach developed by Berry and Kreitman (1993) (see also Verrelli and Eanes 2001). Each site is in turn considered as a “governing” locus and its effect on other loci assessed by Monte Carlo simulation. Details about this method are given in [File S1](#).

Bayesian generalized linear mixed model (Bayenv) analysis: Another approach to detect clinal variation for SNP allele frequencies is to account for population history concomitantly with analysis of environmental correlations, using a Bayesian generalized linear mixed model as implemented in the program Bayenv (Coop *et al.* 2010). This approach has recently been used successfully in humans (*e.g.*, Hancock *et al.* 2011) and in loblolly pine (Eckert *et al.* 2010a) and more details on its implementation can be found in Hancock *et al.* (2008, 2010), Coop *et al.* (2010), and [File S1](#). Briefly, environmental and geographic (*e.g.*, latitude) factors are incorporated as fixed effects, while an estimated variance–covariance matrix of allele frequencies takes into account random effects due to shared population history. A null model of association of SNP frequency with population history is compared to an alternative model that also includes a linear effect of environmental and geographic variables. As a measure of the support for the correlation between SNP frequency and the environmental variables, a Bayes factor (BF) is calculated as the ratio of the posterior probabilities under the alternative and the null models. A BF of 3 is considered to reflect “substantial” evidence for selection and a BF >10 indicates a “strong” support (Kass and Raftery 1995). However, because the null model is a pure drift model, *i.e.*, an unlikely model in most cases, the value of the BF can be biased upward, leading to an excess of false positives. To overcome this problem, we applied the method to all control SNPs to obtain an empirical distribution of BFs from these SNPs (Coop *et al.* 2010; Hancock *et al.* 2010). We scanned all 445 SNPs and picked the outliers according to this empirical distribution. The whole procedure was repeated eight times using eight different draws of the variance–covariance matrix describing population history. The results were averaged across the eight runs.

***F_{st}* outliers**

Since the seminal study of Lewontin and Krakauer (1973), Wright's fixation index, F_{st} , has been extensively used to detect recent selection from population genetics data (e.g., Beaumont and Nichols 1996; Beaumont and Balding 2004; Foll and Gaggiotti 2008). Loci under local selection are expected to show a larger F_{st} value than neutral loci, while loci under balancing selection or purifying selection are expected to show a lower F_{st} value. The available outlier tests using F_{st} can be classified into two groups: tests based on an island model that assumes a common and unique migration pool (Beaumont and Nichols 1996; Beaumont and Balding 2004; Foll and Gaggiotti 2008) and tests based on a hierarchical model that assumes that the population samples can be assigned to groups and that the migration rates are different among demes within groups and between groups [model implemented in Arlequin ver. 3.5 (Excoffier and Lischer 2010)]. If the populations are hierarchically structured, ignoring it can lead to large number of false positives (see Excoffier *et al.* 2009; Narum and Hess 2011). Because an island model seems a reasonable assumption in the present case (see the results of STRUCTURE and hierarchical F_{ST} analyses), we chose to analyze the data with BayeScan (Foll and Gaggiotti 2008; Fischer *et al.* 2011), which has proved to be one of the most reliable methods to detect outliers from the distribution of locus-specific F_{st} (Pérez-Figueroa *et al.* 2010; Narum and Hess 2011). The program assumes that allele frequencies follow a multinomial Dirichlet distribution that arises under a wide range of demographic models. In this Bayesian approach, F_{st} has two components, a population-specific component and a locus-specific component. The alternative model for a given locus is retained when the locus-specific component is necessary to explain the observed pattern of diversity. To account for the fact that we have different numbers of candidate and control SNPs, we used a prior odds ratio of 2, an approximate value of the ratio of control to candidate SNPs. We performed 20 pilot runs with 5000-length and 50,000-step MCMC with additional 50,000 steps of burn-in. Bayes factors as well as posterior probabilities were calculated to indicate how more likely the model with selection is compared to the neutral model. Outliers were identified at 5% and 1% significant levels of posterior probability corrected by the false discovery rate method (Benjamini and Hochberg 1995) as implemented in BayeScan.

Candidate gene expression

As a validation of outliers for environmental correlation with latitude and/or F_{ST} , the expression levels of *PaGI*, *PaPRR7*, *PaFTL2*, and *PaCCA1* were assessed in the aforementioned sample of five Swedish populations. These genes were retained because they either were among the most significant in the Bayenv and BayeScan analyses (*PaGI*, *PaPRR7*, and *PaFTL2*) or had strong physiological evidence as being involved with bud set [*PaCCA1* (U. Lagercrantz, unpub-

lished data)]. Needles were sampled every fourth hour during the last 48 hr of each photoperiodic interval. Twelve seedlings were randomly chosen in each population and needles were collected and pooled. RNA was extracted only from needles sampled during the last 24 hr and on photoperiodic treatments from week 1 to week 6. RNA was extracted according to manufacturer's recommendations, using the STRN250 Spectrum Plant Total RNA kit (Sigma-Aldrich). cDNAs were synthesized from 0.5 μ g total RNA, using Superscript III reverse transcriptase and random hexamer primers. Real-time PCR was performed according to Gyllenstrand *et al.* (2007) or on a MyiQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with the following thermal conditions: 95° for 7 min followed by 40 cycles of 95° for 10 sec and 60° for 30 sec (see Table S2 for a detailed protocol and primer sequences). Each reaction was performed in duplicates containing 12 μ l DyNAmo Flash SYBR Green (DyNAmo Flash SYBR Green qPCR kit; Finnzymes, Espoo, Finland), 0.5 mM of each primer, and 4.75 μ l cDNA (diluted 1:100) in a total volume of 23.75 μ l. Samples were randomized in each step mentioned above, and α -tubulin was used as a reference gene. Each sample was amplified twice with sample positions reversed in the plates. To compare expression levels, the relative expression level of population x under photoperiod i at time-point t is defined as $R_{(xi)}(t) = \Delta CT_{xi}(t) - \Delta CT_{SN}$, where ΔCT is defined as $(CT_{REFERENCE} - CT_{TARGET})$ and ΔCT_{SN} is the average ΔCT value under continuous-light treatment of population SE-58.3 (Saleby). To compare the amplitude between different photoperiod treatments at time-point t , we calculated the squared distance from the mean value within each treatment for every sample as

$$\Delta D^2_{(xi)}(t) = (R_{(xi)}(t) - R_{mean})^2,$$

where $R_{(xi)}(t)$ is the relative expression level and R_{mean} is the mean relative expression level within that photoperiod treatment. For further analysis, we also repeated the whole procedure on the first 24-hr samples under 17.5 hr light/6.5 hr dark and 16 hr light/8 hr dark photoperiod treatments for *PaFTL2* and *PaPRR7*. Nonlinear mixed models with harmonic terms were applied to represent the rhythmic expression patterns, using the nlme library in R (Pinheiro *et al.* 2011). The models are described in detail in Albert and Hunsberger (2005) and Yang and Su (2010).

Results

Bud set and clinal variation

Bud set was measured after each week of photoperiodic treatment and reflects induction some 2 weeks earlier. A clear clinal pattern was observed, even when the southernmost population (SE-58.3) is excluded (Figure 2). Under a 17.5 hr light/6.5 hr dark photoperiod, no individual set bud in the two southernmost populations (SE-58.3 and SE-61.6), ~10–20% of individuals set bud in the two intermediate

populations (SE-62.7 and SE-64.1), and about half of individuals set bud in the northernmost population SE-66.4. If the critical night length for bud set is defined as the time when 50% of plants have set bud, we observe a clinal increase of the critical night length as the latitude decreases.

Linkage disequilibrium

We computed a standard estimate of LD (*i.e.*, r^2) across all individual alleles pooled within each population. Only 560 of 94,673 pairwise comparisons were significant using a chi-square test with Bonferroni correction (P -value = $5.28E-07$), with a total of 157 comparisons with r^2 values >0.3 . The average level of r^2 detected in Norway spruce candidate genes is ~ 0.2 (Figure S3) and is similar to the value observed in other spruce species (Beaulieu *et al.* 2011). Most of these were intragenic and only a few were among genes. Most of the linkage disequilibrium was found in candidate genes, mainly because we had higher SNP density in these compared to control genes, but LD remained limited there too.

Genetic structure

Population genetic structure was not pronounced. The optimal number of clusters calculated with the ΔK method (Evanno *et al.* 2005) is $K = 2$ (Figure 3A and Figure S4A) and the estimated $\alpha > 1.5$, indicating that most individuals are admixed. In line with previous studies (Lagercrantz and Ryman 1990; Heuertz *et al.* 2006; Tollefsrud *et al.* 2008, 2009), the main two clusters consist of populations from the Baltic–Nordic domain (Russian, Finnish, and northern Swedish populations) and of populations completely or partly derived from the Alpine domain (GE-47.0 and SE-58.3). The Bryansk (RU-53.3) population that is located in central Russia (*i.e.*, in one of the putative LGM refugia from which recolonization of northwest Europe started) showed a surprisingly similar clustering pattern to the populations of central Fennoscandia. This was also reflected by a lower F_{ST} value between RU-53.3 and the central Fennoscandia cluster than with others (Table S3). If we choose the mode of posterior probability distribution of the data (Pritchard *et al.* 2000) as a criterion, the optimal number of clusters was $K = 3$ (Figure 3B and Figure S4B). The new cluster was dominated by the four populations from northern Finland that are located at latitudes higher than 66°N .

There was no pattern of isolation-by-distance among all populations (Mantel statistic $r = 0.17$, P -value = 0.143) or among populations when the GE-47.0 (Ruhpolding) and SE-58.3 (Saleby) populations were discarded (Mantel statistic $r = -0.25$, P -value = 0.982). Strong hierarchical structure was not apparent either. The lowest hierarchical level (level 1) was between populations. A second level (level 2) grouped populations into five clusters (GE-47.0, SE-58.3, RU-53.3, central Fennoscandia, and northern Finland) and the highest level (level 3) considered the Alpine domain and the Baltic–Nordic domain. The low hierarchical F -statistic values between these three levels ($F_{\text{level1/total}} = 0.017$,

Figure 2 Percentage of individuals setting bud in five populations under different photoperiodic treatments. “9.5 darkness 1” and “9.5 darkness 2” are week 7 and week 8 within the same photoperiodic treatment (see Materials and Methods).

$F_{\text{level2/level1}} = 0.035$, $F_{\text{level3/level2}} = 0.05$) show that hierarchical genetic structure is negligible and thus applying an island model in the F_{ST} outlier analysis should be reasonable.

Analysis of clinal variation in allele frequencies

Using all of the data, including populations from the Alpine and Baltic–Nordic domains, a total of 54 candidate SNPs, of 137 (39.4%), and 88 control SNPs, of 308 (28.6%), have a regression slope significantly different from zero at the 5% significance level. To reduce the number of positive SNPs simply due to population structure, we removed the three southernmost populations (GE-47.0, RU-53.3, and SE-58.3) and focused on populations located at latitude 61°N and above. A total of 92 of 445 SNPs, 34 (24.8%) of which were candidates and 58 (18.8%) of which were controls, showed a significant linear regression with latitude at the 5% significance level (Table 2 and Figure 4). Finally, when we removed the four northernmost populations and kept only populations from central Fennoscandia (latitudes ranging from 61°N to 65°N), there were only 28 significant SNPs including 11 from candidate genes.

The adjusted R^2 of regression of SNP frequency on latitude was estimated to represent how much of the variance in frequency could be explained by latitude and test for enrichment of the candidate SNPs among significant SNPs at the 5% significance level. In all three data sets there was no enrichment in candidate SNPs among significant ones.

Observing that there are 92 SNPs in the data set without the three most southern populations whose allele frequencies are significantly correlated with latitude and the presence of LD among candidate SNPs, we tested whether clinality at one site affects that of flanking SNPs. A Monte Carlo simulation approach was used for this purpose and

Figure 3 Clustering analysis conducted in STRUCTURE. (A) $K = 2$; (B) $K = 3$.

only the 34 significant candidate SNPs were investigated as they showed higher levels of LD (Figure S5). Nonsignificant SNPs can be also affected by nearby variants under selection but they were ignored since they do not exhibit clinal variation and were unlikely to explain clinal variation at other segregating sites. Two broad patterns were apparent. One pattern involved clinality being driven by LD within and among genes, such as that observed at PaMFTL1 SNPs. In contrast, most of the effect of clinality for SNPs in the promoter of PaFTL2 came from within the promoter itself, as was already suggested by the pattern of LD. The analysis also showed a group of SNPs whose effect could be explained by a small number of other SNPs such as PaMFTL1_3658, PaFTL2pr_1951, PaPHYP_RIII274, PaGI_F2_9_987, and PaPRR7_F3_104. These SNPs had a higher level of clinality than others and usually showed stronger signals in the two other tests (see results below and Table 2).

Bayesian generalized linear mixed model (Bayenv) analysis

The average BF estimated from the candidate SNPs was significantly higher than the one estimated from control SNPs (average BFs = 3.26 and 1.48, respectively; Wilcoxon two-sample test P -value = 0.0083). More importantly, there was a significant enrichment of candidate SNPs in the upper tail of the empirical distribution of BFs. The ratio at the 10% tail ($BF \geq 2.7$) was 0.87 (27/31, candidate to control SNPs) and increased to 1.12 (18/16) in the 5% tail ($BF \geq 4.6$) and

~ 1.75 (7/4) in the 1% tail ($BF \geq 10.4$) (bootstrap <1% for all tails, see Table 2). To remove the possible effect of control loci that were potentially under selection, we then excluded 14 control SNPs that were outliers in more than one of the tests [linear regression, Bayenv, and F_{ST} outliers (see below)] and reconstructed the null model. This caused an increase of BF estimates in the upper 25% tail but the candidate outliers and the enrichment ratio remained the same (0.9, 1.06, and 2 at 10%, 5%, and 1% tails, respectively).

F_{ST} outliers

The average value of F_{ST} across SNPs was 0.05. Candidate SNPs had significantly higher F_{ST} values than control SNPs (mean F_{ST} = 0.056 and 0.047, respectively; Wilcoxon rank sum test P -value = 0.00026). BayeScan identified 29 outliers at the 5% significance level [corrected false discovery rate (FDR) test], 16 of which were from candidate loci. At the 1% significance level, 11 candidate SNPs and 6 control SNPs were identified as outliers (Figure 5). Fisher's exact test indicated a significant enrichment of candidate SNPs in these tails (P -values = 0.0064 and 0.0054, respectively). At these significance levels, all outliers had high F_{ST} values and therefore were putatively under positive selection. The exception was PaKN2b_1157 and two other control SNPs that had low F_{ST} values (~ 0.020).

Summary of the analysis of SNP variation

We combined the results from all of the analyses above (Figure S6) to obtain a short list of loci with evidence of

Table 2 Summary of the SNPs detected by the different approaches

SNP	Clinality: R^2	MC sampling: no. SNPs affecting the SNP	BayeScan			Annotation		
			Affected by	$\text{Log}_{10}(\text{P.O.})$	F_{ST}	Bayenv BF	AA change	Nucleotide
PaPHYN_RIII185	0.256	25	7	−0.360	0.043	0.710		
PaPHYN_RIII88	0.510	0	12	−0.427	0.051	4.276		
PaPHYO_RIII510	0.213	33	5	−0.353	0.054	1.938		
PaPHYP_RIII274	0.404	0	14	2.743	0.139**	25.175**		
PaPHYP_RIII76	0.408	1	10	−0.409	0.045	1.879		
PaMFTL1_1050	0.316	23	9	2.467	0.129**	5.489*		
PaMFTL1_2091	0.383	3	11	2.013	0.127**	11.692**		
PaMFTL1_2136	—	—	—	0.126	0.072	5.813*		
PaMFTL1_2215	0.285	31	9	−0.492	0.048	1.263		
PaMFTL1_3441	0.359	18	7	1.788	0.122**	12.817**		
PaMFTL1_3658	0.555	0	12	0.333	0.080	6.414*		
PaMFTL1_802	—	—	—	1.469	0.127*	6.540*		
PaFTL2pr_1560	0.419	6	9	−0.417	0.050	0.617		
PaFTL2pr_1824	0.493	0	15	−0.347	0.053	3.300		
PaFTL2pr_1951	0.641	0	14	1.540	0.121**	18.346**		
PaFTL2pr_2173	0.459	6	13	0.850	0.101*	5.254 *		
PaFTL2pr_2509	0.441	2	13	1.915	0.133**	16.667**		
PaFTL2pr_2694	0.387	7	13	1.159	0.108*	5.752*		
PaFTL2pr_2790	0.396	11	12	1.284	0.112*	4.963*		
PaGI_F2_9_1470	0.543	0	7	0.596	0.090	2.752	His to Tyr	CAT to TAT
PaGI_F2_9_987	0.479	1	8	1000	0.170**	117.407**	His to Tyr	CAT to TAT
PaGI_F6_8_1111	0.244	1	6	−0.061	0.036	0.627		
PaHB3_385	0.349	0	8	−0.116	0.036	0.582		
PaKN2a_253	0.240	33	6	−0.411	0.043	0.557		
PaKN2b_1157	—	—	—	0.702	0.020*	0.592		
PaKN3a_211	0.377	0	12	−0.521	0.045	0.873		
PaKN4b_242	0.251	33	8	0.415	0.088	47.522**		
PaCCA1_1302	0.288	22	7	−0.381	0.043	0.533		
PaPRR1_1992	—	—	—	−0.529	0.047	4.884*		
PaPRR1_2990	—	—	—	−0.503	0.048	4.871*		
PaPRR1_3301	—	—	—	−0.511	0.048	4.721*		
PaPRR3_F1_2570	0.380	0	7	−0.319	0.055	3.244		
PaPRR3_F1_2978	—	—	—	1.722	0.123**	2.568		
PaPRR3_F2_331	0.309	25	8	−0.504	0.048	4.213		
PaPRR3_F2_481	0.354	1	8	−0.369	0.055	3.009		
PaPRR7_F1_1505	0.455	0	11	−0.237	0.060	2.965		
PaPRR7_F1_771	0.683	2	11	2.467	0.126**	3.842		
PaPRR7_F2_417	0.230	33	7	−0.374	0.053	1.768		
PaPRR7_F2_534	0.673	2	11	1000	0.138**	4.458	Ser to Thr	TCT to ACT
PaPRR7_F3_104	0.689	2	11	1000	0.161**	9.906*		
PaZTL_793	0.352	7	7	−0.343	0.041	0.677		

See the main text for details on the different approaches. * $P < 0.05$; ** $P < 0.01$. $\text{Log}_{10}(\text{P.O.})$ (P.O., posterior odds): 0.5–1, substantial evidence; 1–1.5, strong evidence; 1.5–2, very strong; >2, decisive evidence. Bayes factors: 3.2–10, substantial evidence; 10–100, strong evidence; >100, decisive evidence.

clinal variation and/or selection (Table 2). Most of the SNPs displaying strong signals in all our analyses came from five genes: PaMFT1, PaFTL2, PaGI, PaPRR7, and PaPHYP. One particularly interesting SNP was PaFTL2pr_1951, which represents a recent mutation that does not exist in Brewer spruce or in the RU-53.3 (Bryansk) population, both of which served as outgroups, or in populations from the Alpine domain [GE-47.0 (Ruhpolding) and SE-58.3 (Saleby)]. The clinal pattern in allele frequency at PaFTL2pr_1951 cannot be explained by any other SNP, but PaFTL2pr_1951 affects the clinal variation at 14 other significant candidate SNPs. Previous studies on expression of *FT-Like* genes in Norway spruce have shown that the expression level of PaFTL2 correlates with bud set. Expression is induced by

night lengths exceeding the population-specific critical night length for bud set for trees from Romanian and Arctic populations, respectively (Gyllenstrand *et al.* 2007). The single-nucleotide mutation in the PaFTL2 promoter might affect the divergent expression patterns in genotypes with varying critical night lengths. Other strong outlier SNPs were located in putative circadian clock genes (PaPRR7 and PaGI) that are likely regulators of PaFTL2 expression and bud set (A. Karlgren, N. Gyllenstrand, and U. Lagercrantz, unpublished data). PaPRR7_F2_534 is a nonsynonymous substitution causing an amino acid replacement from serine to threonine (TCT to ACT in nucleotides) and was found to be associated with two other SNPs, PaPRR7_F1_771 and PaPRR7_F3_104 (in intron and 3'-UTR regions, respectively).

Figure 4 Examples of candidate SNPs that show a significant regression of the transformed allele frequency on latitude.

PaGI_F2_9_987 and *PaGI_F2_9_1470* are linked and both cause an amino acid replacement from histidine to tyrosine (CAT to TAT in nucleotides). Using results from the NCBI BLASTP 2.2.25+ tool (Altschul *et al.* 1997), histidine is likely the conserved state, as all 64 hits from 47 species with high similarity ($e\text{-value} < 6e-95$) had a histidine in this position.

Candidate gene expression

For a subset of candidate genes, we compared the relative expression levels among photoperiodic treatments using the pairwise Wilcoxon rank sum test implemented in R (R Development Core Team 2011). The mean expression level of *PaFTL2* across all populations increased continuously as the night length increased from near 0 in constant light to 2.7 at night length of 3.5 hr and to 6.0 at night length of 8 hr. In the two southernmost populations (SE-58.3 and SE-61.6), *PaFTL2* was significantly upregulated at night length of 6.5 hr compared to the expression level under constant light ($P\text{-value} < 0.01$, FDR corrected). In the two northernmost populations (SE-64.1 and SE-66.4), this change happened

for a night length of 5 hr ($P\text{-values} < 0.01$ and < 0.05 , respectively). This change might happen even earlier in SE-66.4 as the $P\text{-values}$ are 0.052 for night lengths of 2 hr and 3.5 hr. In population SE-62.7, there was a significant increase in expression of *PaFTL2* already at 2 hr night length, which is earlier than expected on the basis of previous results. Except for SE-58.3, all populations showed a second increase in expression level when the night length reached 8 hr compared to the first increase in expression level ($P\text{-value} < 0.05$ for SE-62.7 and < 0.001 for the other three). We therefore compared the relative expression level of *PaFTL2* among populations under a night length of 6.5 hr or 8 hr. There is a significant difference ($P\text{-value} < 0.001$) in *PaFTL2* expression between SE-58.3 (3.76) and other populations (5.14 on average) for a night length of 6.5 hr (Figure 6). Furthermore, when the night length is 8 hr, the mean expression levels of the five populations are significantly differentiated (SE-58.3, 4.11; SE-61.6, 6.22; SE-62.7, 6.20; SE-64.1, 6.70; and SE-66.4, 6.73; $P\text{-value} < 0.05$) and linearly correlated with latitude ($P\text{-value} = 0.048$, adjusted $R^2 = 0.75$, Figure 7).

Figure 5 Analysis of F_{ST} outliers using BayeScan (Foll and Gaggiotti 2008). Estimates of F_{ST} for candidate SNPs (black circles) and background SNPs (white circles) are plotted against the logarithm of the posterior odds.

Unlike *PaFTL2*, the expression levels of *PaCCA1*, *PaGI*, and *PaPRR7* were similar across photoperiod treatments, and differences were not statistically significant. These genes are presumably located in the circadian clock (*PaCCA1*, *PaGI*, and *PaPRR7*) or related to it (*PaFTL2*) and their expression level is expected to show a diurnal expression rhythm. To measure the amplitude of their rhythm, we calculated the squared distance from the mean level of relative expression in each photoperiod treatment. There was an increase of amplitude in the 16 hr light/8 hr dark photoperiod in all five populations in *PaPRR7* (P -value < 0.05) compared to that in constant light. Furthermore, we represented their cycle trends using harmonic regression with one or two periods. The predicted curves of *PaPRR7* and *PaGI* showed nice diurnal rhythms with a periodicity of ~ 24 hr (see Figure 6 and Table S4 for all estimated parameters), while neither *PaFTL2* nor *PaCCA1* fit the autoregressive model well and showed no clear diurnal rhythms.

Discussion

In this study, we have used different approaches to identify SNPs in candidate genes that are potentially associated to the clinal variation observed for bud set in Norway spruce. The combined approaches identified a promising set of SNPs, in particular in the promoter of the *PaFTL2* gene. The latter was furthermore accompanied by clinal variation in expression level. Below we discuss some caveats of the different approaches, how they relate to each other, and how their joint use can shed light on the genetic control of bud set and local adaptation. In particular, we discuss the effect of population histories on the interpretation of clinal variation. Finally, we briefly outline the implications of the present results for the design of future association-mapping studies for traits showing strong clinal variation and give some suggestions for future extensions of the current study.

Ascertainment bias

The SNPs analyzed here were discovered using three different discovery panels. All candidate SNPs originated

from a large discovery panel (~ 48 chromosomes), while the bulk of the control SNPs come from smaller discovery panels (12 chromosomes in the CRSP resequencing panel and 24 chromosomes in the Arborea genotyping panel). Albrechtsen *et al.* (2010) reported that the size and the geographic distribution of the discovery panel could lead to a bias in the site-frequency spectrum. They also showed that as long as the discovery panel size is not too small (< 4 chromosomes), estimates of F_{ST} are rather robust to ascertainment bias since the bias has a similar effect on the total- and among-population variances. In our data, we observed a deficit of low-frequency alleles in the control loci (mean allele frequencies = 0.33 and 0.24 for control and candidate SNPs, respectively, P -value $< 2.2e-16$ in two-samples t -test). As expected, the control SNPs have significantly higher heterozygosity than candidate SNPs (mean heterozygosities = 0.29 and 0.25 for control and candidate SNPs, respectively, P -value = 0.0238). F_{ST} values among populations are, however, of the same order of magnitude as estimates obtained through analysis of sequence data (mean F_{ST} 's = 0.056 and 0.047, for candidate and control, respectively, and 0.040 between northern and southern Sweden in Heuertz *et al.* 2006) although it should be pointed out that the two studies have many SNPs in common.

The control SNPs were also used to build a covariance matrix between populations for the null model used by Bayenv. The covariance matrix can be viewed as a model-based, population-specific estimate of F_{ST} (Nicholson *et al.* 2002; Coop *et al.* 2010). Hence, in both the Bayenv and the BayeScan analyses, the results should not have been affected too strongly by ascertainment bias, and selection should indeed be the major cause of the substantial difference in F_{ST} estimates between control and candidate SNPs. This is supported by the analysis of the site-frequency spectrum for control SNPs and candidate SNPs in the total data (Figure S7A) and for the significant SNPs detected in the three main analyses: clinal variation (Figure S7B), Bayenv analysis (Figure S7C), and the BayeScan analysis (Figure S7D). The site-frequency spectrum for each of those sets of SNPs did not show any specific structure, and there was

Figure 6 Mean level per population of the relative RNA expression of four putative photoperiodic genes under different photoperiodic treatments. Autoregressive models were used to obtain the curves, except for PaCCA1 for which the fit was poor and different points were just joined by a line. (A–D) At the bottom, white rectangles represent light periods and black rectangles represent dark periods. (A) PaPRR7; (B) PaGI; (C) PaFTL2; (D) PaCCA1.

no clear overrepresentation of the candidate SNPs in the lowest allele-frequency classes.

Population history, selection from standing variation, and local selection

A major issue when trying to identify SNPs associated to local adaptation is to account for demographic processes that can leave the same signature (Väsemegi 2006; De Carvalho *et al.* 2010; Savolainen *et al.* 2011). Norway spruce, as well as many other plant and animal species, recolonized northern Europe after the LGM along at least two main recolonization routes (Giesecke and Bennett 2004; Tollefsrud *et al.* 2009). Thus, the current northern European spruce forests were established during a rather short evolutionary period and from different sources. This is indeed what our data suggest. There are three main genetic clusters, but with the exception of the population from Germany and the four populations from northern Finland, no populations can be assigned entirely to any of these three clusters. In the case of population SE-58.3, the presence of a group of individuals exhibiting a close similarity to individuals from the German population likely reflects the use of seeds imported from Germany and Belorussia in afforestation over the last century. Apart from Germany and the four populations from northern Finland, the pattern in populations ranging from 60°N to 65°N suggests a modest contri-

bution from the Alpine domain (the blue cluster in Figure 3), as well as more substantial contributions from two eastern LGM refugia (green and red clusters in Figure 3). One of these two refugia, the one associated with populations from northern Finland, was probably located at a higher latitude than the other, a possibility suggested by recent paleogeographic studies (Väliiranta *et al.* 2011). The green cluster could also reflect the survival of trees in Scandinavia or close to it during the LGM (Öberg and Kullman 2011), but if those scattered refugia had contributed to present-day populations, the similarity and the genetic closeness of the Russian population (RU-53.3) to populations from central Fennoscandia (Figure 3B and Table S3) might be difficult to explain since it would run counter to the general direction of recolonization inferred from both genetic (Lagercrantz and Ryman 1990) and paleoecological studies (Giesecke and Bennett 2004).

Independently of the exact geographical origin of the source populations, the spread of populations together with the existence of diverse origins could lead to serial founder effects and secondary contact, which in turn may result in neutral polymorphisms showing clines in allele frequencies (Novembre and Di Rienzo 2009). Two recent studies have investigated the possibility that secondary contacts and admixture could play a role in local adaptation in Scandinavian tree species. In Scots pine (*P. sylvestris*), Savolainen *et al.*

Figure 7 Correlation of the mean level of expression of PaFTL2 under a night length of 8 hr with latitude (P -value = 0.048, adjusted R^2 = 0.75).

(2011) detected no admixture zone and speculated that adaptation occurred during the colonization process from standing variation. De Carvalho *et al.* (2010) did detect admixture in European aspen (*Populus tremula*) from central Sweden and argued that admixture facilitated adaptation from standing variation. Both studies differed from ours in major ways. The inference of population structure in *P. sylvestris* was based on only three populations and a limited number of markers. Therefore, it may not have had the power to detect a putative admixture zone. The study of De Carvalho *et al.* (2010) was based on seven populations, only one of them located in Scandinavia and the others being far from it. Admixture in European aspen was inferred in this single Swedish population located at 63°N, possibly reinforcing the impression of selection from standing variation.

In Norway spruce, while we did not observe any evidence of isolation-by-distance (Mantel test, P -value > 0.95), we did detect evidence of secondary contacts and admixture. To tell apart selection from demography we used a three-step approach. First, we used simple regression analysis of allele frequencies on latitude for differing subsets of the data. The number of SNPs showing clinal variation decreased sharply when populations from northern Finland were not considered, suggesting that part of the clinal variation reflected population structure. Since we did not correct for demography in these regression analyses, no enrichment in candidate genes was observed in the tails when the adjusted R^2 statistics were considered. This simply means that demography is, on the average, affecting all SNPs equally across the genome, irrespective of their status (candidate vs. noncandidate), and that demography needs to be accounted for if one wants to identify SNPs also affected by

selection. Second, since population structure is not accounted for in the regression analysis, we used a Bayesian generalized linear mixed model (Bayenv) on all populations (Hancock *et al.* 2008, 2010, 2011; Coop *et al.* 2010). This led us to discard many SNPs that had high R^2 in the standard cline analyses, suggesting that the strong clinal variation at those SNPs likely reflects population structure. However, since we also observed an enrichment of candidate SNPs when population structure was accounted for, it would therefore seem that the data also reflect selection, possibly from standing variation, rather than from new mutations. The latter is supported by the fact that most of the alleles that show significant SNPs are already present in the Russian population, which is close to the putative ancestral refugia. One of the most interesting SNPs, however, is PaFTL2pr_1951, which was absent in Brewer spruce in the Russian population and in the German population and therefore appears to be a new mutation. Third, we tested for F_{ST} outliers to assess whether the enrichment in candidate SNPs that we detected with Bayenv was due to selection in past environments or since the populations colonized Scandinavia. In contrast to results in European aspen (Ma *et al.* 2010), we did detect significant SNPs when we used an F_{ST} -outlier approach, suggesting the presence of some amount of local selection. Furthermore, there was a good correspondence between the Bayenv and the F_{ST} -outlier approaches, with 12 SNPs picked by the two methods and 4 and 6 identified uniquely by Bayenv and the F_{ST} -outlier approach, respectively. Ma *et al.* (2010) argued that extensive long-distance gene flow in wind-pollinated forest trees (e.g., Robledo-Arnuncio 2011) will reduce population genetic structure and will also overwhelm selection and limit our ability to discover selection signal by erasing peaks of genetic differentiation (Ma *et al.* 2010). In our study, local selection seems to have been strong enough to generate extreme F_{ST} values at certain loci so that they emerge as outliers in the analysis and, furthermore, we also observe a significant enrichment of candidate SNPs among the significant ones. Prunier *et al.* (2011) obtained similar results in black spruce.

Differentiation at quantitative trait loci

Some authors (Latta 1998; Le Corre and Kremer 2003; Kremer and Le Corre 2011) have argued that low values of F_{ST} will be observed even at loci underlying quantitative traits and that this reflects a decoupling between the polymorphism of a quantitative trait and the polymorphism of its underlying quantitative trait loci. For instance, in the model of Kremer and Le Corre (2011), natural selection first acts on beneficial allelic associations at different loci in different populations and only significantly affects allelic frequencies at individual loci at later time points. The high F_{ST} values obtained for significant outlier SNPs (the mean F_{ST} of outliers is 0.13 if the only value that was in the lower tail of the distribution is removed, Table 2) are also in contrast to results presented by Kremer and Le Corre (2011), where

candidate SNPs and control SNPs exhibited similar values of F_{ST} . We believe that this is simply due to the fact that most of the candidate SNPs used in Kremer and Le Corre (2011) may actually not be associated to the studied quantitative traits. Nonetheless, as in Namroud *et al.* (2008), Kremer and Le Corre (2011), and Prunier *et al.* (2011), F_{ST} values at candidate gene SNPs were still much smaller than Q_{ST} values.

Expression patterns of candidate genes

Most of our putative candidate genes were chosen as representatives of the photoperiodic pathway and include putative photoreceptors, circadian clock genes, and genes acting downstream of these genes. Even though the function of most of these candidate genes in Norway spruce is still poorly characterized, many of the significant SNPs were located in genes that are logical candidates on the basis of current knowledge. One such example is the set of significant SNPs located in the *PaPHYB* gene. Physiological experiments have shown that genotypes from high latitudes do not respond to exposure to red light during an artificial long night and thus set buds, in contrast to genotypes from more southern latitudes (Clapham *et al.* 1998; Gyllenstrand *et al.* 2007). On the basis of sequence homology, *PaPHYB* corresponds to *PhyB* in angiosperms, which is the receptor that is predominantly responsible for responsiveness to red light (Schneider-Poetsch *et al.* 1998), and clinal variation at *PhyP* SNPs was observed in Scots pine (Garcia-Gil *et al.* 2003). Significant SNPs were also identified in putative circadian clock genes, in particular in *PaGI* and *PaPRR7*. Our unpublished data support that these genes are indeed part of the Norway spruce circadian clock and furthermore show divergent expression patterns in genotypes from extreme northern and southern latitudes, in particular, divergent responses to night breaks (A. Karlgren, N. Gyllenstrand, and U. Lagercrantz, unpublished data). These results also agree well with studies in Sitka spruce and poplar where this category of genes was found to exhibit clinal variation and be associated with bud set (Holliday *et al.* 2010; Ma *et al.* 2010; Hall *et al.* 2011; Hsu *et al.* 2011).

If enhanced genetic structure in these candidate genes is truly caused by selection, we might expect a divergence in gene expression levels among alleles. As the circadian clock is composed of multiple integrated feedback loops, mutations in both regulatory and coding parts could affect the transcriptional patterns of multiple clock genes. When analyzing gene expression we used a bulk-sampling strategy, with each population being represented by 12 randomly chosen individuals. Thus, the expression level should reflect the effect of each allele weighted by its frequency. This could undermine the estimation of the true expression divergence between the two alleles, as the effect of a slight allele frequency shift caused by weak selection may not be detected. In this study, the expression levels of *PaPRR7*, *PaCCA1*, and *PaGI* show very subtle differentiation along the latitudinal gradient. However, this marginal difference

might still cause significant expression changes in downstream genes such as *PaFTL2* and divergent bud set and growth cessation along the latitude in phenotype.

Our previous studies have shown that *PaFTL2* expression is controlled by photoperiod and is correlated to bud set under various experimental conditions. Additionally, *PaFTL2* expression differs between genotypes from extreme northern and southern latitudes (Gyllenstrand *et al.* 2007). In the present study, we found a weak but significant latitudinal cline in the expression of *PaFTL2* correlated to bud set in a more limited part of the latitudinal range. In light of these data, the identification of several significant SNPs in the promoter of *PaFTL2* is very encouraging, especially as some are located in or close to putatively important binding sites. Analyses using “Plant promoter analysis navigator” [PlantPAN (Chang *et al.* 2008)] and “RNAhybrid” (Krüger and Rehmsmeier 2006) indicate that the *PaFTL2pr_1951* SNP is located in the 3' end of a putative miRNA-target binding site of a member of the miR169 and/or miR399 families, which are important transcriptional regulators in plants (reviewed by Bartel 2004), and is located next to the binding site of an “E2F Consensus Element” transcript factor that might cause an affinity change.

Implications

One of the main aims of the statistical methods developed for association studies is to decrease the number of false positives due to population structure. This, however, could lead to false negatives if population history and differences in allele frequency at causal SNPs covary as may be the case in the present study (Atwell *et al.* 2010; Eckert *et al.* 2010b). As pointed out by Atwell *et al.* (2010, p. 630), “plants from northern Sweden will tend to share cold-adaptive alleles at many causal loci as a result of selection, and marker alleles genome-wide as a result of demographic history.” Atwell *et al.* (2010) were referring to *A. thaliana* but our results and those of De Carvalho *et al.* (2010) suggest that this may also fit well to Norway spruce and European aspen. In both species, the strong latitudinal clines observed at many SNPs may reflect the presence of different glacial refugia as well as different selection pressures.

In the present study, we have found evidence of clinal variation at SNPs in candidate genes, using populations from a limited part of the Norway spruce natural range. We also observed that *PaFTL2* expression varied along the latitudinal cline. Demonstrating that the identified SNPs are truly causal for bud set, however, will remain a daunting task in a nonmodel species like Norway spruce. Ralph and Coop (2010) showed that parallel adaptation can occur within the same species, something that is of major interest in species like Norway spruce that have a fairly large global effective population size and a distribution that may allow for several clines to be established. Independent clines have been extensively used to study patterns of adaptation in *Drosophila*, and this seems to be a fruitful approach for detecting loci underlying local adaptation (e.g., Oakeshott

et al. 1982; Turner *et al.* 2010). To validate the SNPs and loci identified in the present study, it would be highly interesting to test whether clinal variation at the loci identified here is also observed in other regions of the same species with a different history or in other species.

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Supporting Information

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Disentangling the Roles of History and Local Selection in Shaping Clinal Variation of Allele Frequencies and Gene Expression in Norway Spruce (*Picea abies*)

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File S1
Supporting Text

Details on the implementation of the Berry and Kreitman (1993) approach.

The method works as follows. Each site is in turn considered as a “governing” locus and its effect on other loci assessed by simulation. First the observed allele frequency at the “governing” locus in each population is kept constant and simulates the allele frequencies of affected locus using binomial sampling around the expected values. For example, consider two sites X (governing) and Y (target). We hold the frequency of allele A at site X constant in each population and recalculate the frequency of allele B at site Y. Suppose that we have a pooled data set of 500 individuals. The overall frequency of allele B at site Y is 50% and allele B was observed in 20% of the individuals carrying allele A at site X and in 30% of the individuals carrying another allele. If, in population i , the allele A is present in 30 out of 50 individuals at site X, then the expected frequency of allele B at site Y in population i can be calculated as $(0.2 \times 30 + 0.3 \times 20) / 50 = 0.24$. We then perform a binomial sampling around this expected allele frequency for population i and repeat it for each population and each target locus to obtain 1000 simulated data sets. These 1000 simulated frequencies data sets for each population result in 1000 R^2 values of the linear regression of allele frequencies on population latitude at the target loci based on the observed allele frequency at the governing locus. After 1000 iterations, the R^2 values are sorted to obtain a confidence interval for the expected R^2 distribution. If the observed R^2 falls within the 95% confidence interval, the observed geographic variation at the target locus is simply explained by its linkage disequilibrium with the governing locus.

Bayesian generalized linear mixed model analysis (Coop et al. 2010).

COOP *et al.* (2010) developed a Bayesian generalized linear mixed model to test the correlation between SNP variation and environmental variables while accounting for population structure. This approach has recently been used successfully in humans (e.g. HANCOCK *et al.* 2011) and in loblolly pine (ECKERT *et al.* 2010). In COOP *et al.* model, environmental factors, including geographic parameters, such as latitude, are incorporated as fixed effects while an estimated variance-covariance matrix takes into account all random effects due to shared population history. In its assumption, the population frequencies randomly deviate from the ancestral frequency (ϵ_i) due to genetic drift, and the populations should co-vary in their deviation because of shared ancestry and gene flow. One also assumes that the transformed allele frequency at the i th SNP follows a multivariate normal distribution and the null model is defined as: $P(\theta_i | \Omega, \epsilon_i) \sim N(\epsilon_i, \epsilon_i(1-\epsilon_i)\Omega)$, where θ_i is the transformed allele frequency across populations, and Ω is the covariance matrix that is shared by all loci. We performed 4 runs with 100 million steps of MCMC each in order to make sure that Ω is convergent and reflects the true population structure.

The alternative model includes the correlation between allele frequency at each SNP and the population latitude or other environmental variables (Y) by adding a fixed effect to the mean of the multivariate normal distribution: $P(\theta_i | \Omega, \epsilon_i) \sim N(\epsilon_i + \beta Y, \epsilon_i(1-\epsilon_i)\Omega)$, where β is the linear effect of the environmental variable Y . As a measure of the support for the correlation a Bayes factor (B.F.) is calculated as the ratio of the posterior probabilities under the alternative and the null models. A single draw of Ω estimates under the null model across all SNPs was used for each run of correlation test. We scanned all 445 SNPs and picked the outliers according to the empirical distribution generated by sorting the BF to calculate a significant level for each SNP. The whole procedure was

repeated 8 times using 8 different draws of Ω . The results were averaged across the 8 runs. More details on the model can be found in COOP *et al.* (2010), HANCOCK *et al.* (2010a) and HANCOCK *et al.* (2010b).

Figure S1 The general outline of this study.

Figure S2 Photoperiodic treatments and sampling schedule for phenotype and RNA expression studies

Figure S3 Pairwise linkage disequilibrium estimated using r^2 . a. Baltico-Nordic domain populations b. German population Ruhpolding c. Southern Swedish population Saleby

Figure S4 The optimal K choose using a. log posterior probabilities and b. ΔK

Monte Carlo Sampling at Significant Clinal Candidate Loci

Figure S5 Summary of the Monte Carlo sampling for the site-by-site analysis. Only candidate SNPs that exhibited a significant regression with latitude are shown. Shaded boxes represent the significant clinal variation at the target loci can be explained by geographic variant at the governing loci.

Figure S6 A Venn diagram summarizing the results from the three different tests applied to the data. The figures are ratios of candidate to control SNPs in the different tests and combination of tests (at 5% significant level). The numbers in non-overlapping areas are the results obtained when only one method was applied while the numbers in the overlapping areas are the results when combining the results from the different methods (see Table 2 in text for details regarding the number of outliers).

Figure S7 Allele frequency spectrum of candidate (dark) and control (grey) loci. a. Total SNP data set used in this study. b. SNPs shown significant clinal variation analysis. c. Outlier SNPs at 5% tail of B.F. distribution from Bayesian generalized linear mixed model. d. Outlier SNPs at 5% tail of log(P.O.) distribution from BayeScan analysis

Table S1 Description of the candidate SNPs and control genes used in this study

Table S1 is available for download at

<http://www.genetics.org/content/suppl/2012/04/27/genetics.112.140749.DC1> as an Excel file.

Table S2**cDNA synthesis:**

The tubes containing Mix1 (Table S2-1, RNA integrity and concentration was measured using both QIAxcel and NanoDrop machines) were incubated in 70 °C for 10 minutes and then kept on ice. Mix2 (Table S2-2) was added to each tube to a total volume of 16 µl / tube and incubated overnight in 42 °C.

Real-Time PCR:

Real-Time PCR mix (Table S2-3) was denatured in 96°C for 7 minutes followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds.

Table S2-1 cDNA synthesis Mix1

RNA:	0.5 µg
Random primers (0.3 µg/µl, Invitrogen)	1.0 µl
RNase free water	add to 9.0 µl / reaction

Table S2-2 cDNA synthesis Mix2

FSB (5X)	3.0 µl
DTT (0.1 mM)	1.5 µl
dNTP Mix (10 mM)	1.0 µl
Super Script III Reverse Transcriptase	0.5 µl
Total sum	6.0 µl

Table S2-3 Real-Time PCR mix

Sybr Green	12 µl
Primer_F (10 µM)	1.2 µl
Primer_R (10 µM)	1.2 µl
water	4.6 µl
cDNA (diluted 1/100)	4.8 µl

Table S2-4 Real-Time PCR primer sequences

Primer Name	Sequence
PaMyb2_RT_new_I_Fwd	ACGAAGCCATGCTCAGAAGT
PaMyb2_RT_new_I_Rev	GGGATAAGGATGCCTTGGTT
PaPRR7_RT_Fwd	CCGTCAGAAAAGGAAAGAGAGAT
PaPRR7_RT_Rev	GTCACCCTTGGTCGTTGTTC
PaFTL2_RT_Fwd	CGATGTTGGAGGAGACGAC
PaFTL2_RT_Rev	GCATAAGCTCCCGTCCAAA
PaGI_II_RT_Fwd	GGCAGAAGGGCTATGGAA
PaGI_II_RT_Rev	ATGTACTAAGTGC GCGGACA
Alpha-Tubulin_Fwd	GGCATACCGGCAGCTCTTC
Alpha-Tubulin_Rev	AAGTTGTTGCCGGCGTCTT

Table S3 F_{ST} values between outlying populations and populations from Scandinavia

	GE-47.0	RU-53.3	SE-58.3	Central Scandinavia	Northern Scandinavia
GE-47.0	0				
RU-53.3	0.09841	0			
SE-58.3	0.01574	0.05008	0		
Central Scandinavia	0.09324	0.02924	0.03486	0	
Northern Scandinavia	0.21465	0.09164	0.13825	0.04971	0

Table S4 Parameters of the expression study

Population	Gene	Photoperiod	num_period(n)	Period(hours)	Frequency	Mean	Amplitude1	Phase1	Amplitude2	Phase2	P value	Adjusted R ²
Saleby	PaPRR7	6.5-h dark	2	24.0883029	0.9963342	0.2572301	-1.171126	0.3764416	-0.8575932	0.2843278	1.08E-05	0.8539805
Istevallen	PaPRR7	6.5-h dark	2	24.0883029	0.9963342	0.3794638	-1.179671	0.3468512	-0.8575984	0.2875196	3.20E-08	0.9540118
Hogmansbod	PaPRR7	6.5-h dark	2	24.0883029	0.9963342	0.4407237	-1.183953	0.3320218	-0.857601	0.2891152	3.08E-06	0.8860118
Jamtland	PaPRR7	6.5-h dark	2	24.0883029	0.9963342	0.3763569	-1.179453	0.3476044	-0.8575982	0.2874372	8.24E-05	0.7819624
Norrland	PaPRR7	6.5-h dark	2	24.0883029	0.9963342	0.3814307	-1.179809	0.3463717	-0.8575985	0.2875698	1.34E-05	0.8475043
Saleby	PaPRR7	8-h dark	2	24.46223596	0.9811041	-0.106884847	-1.904577	0.288863	-1.252201	0.2351993	2.02E-10	0.9832656
Istevallen	PaPRR7	8-h dark	2	24.46223347	0.9811042	-0.065075785	-2.054817	0.2553925	-1.49951	0.2244521	3.29E-08	0.9537639
Hogmansbod	PaPRR7	8-h dark	2	24.46223347	0.9811042	-0.120554783	-1.935065	0.2641766	-1.377222	0.2315329	2.23E-07	0.9322808
Jamtland	PaPRR7	8-h dark	2	24.46223596	0.9811041	0.19784703	-1.90541	0.2839268	-1.162195	0.2278678	8.95E-09	0.9643297
Norrland	PaPRR7	8-h dark	2	24.46223347	0.9811042	0.009672734	-2.132017	0.2690722	-1.492159	0.2211576	1.41E-08	0.9609403
Saleby	PaFTL2	6.5-h dark	2	32.72120592	0.7334693	3.936021	1.10196	0.6157213	0.4071825	0.61158736	0.030203166	0.3279688
Istevallen	PaFTL2	6.5-h dark	2	18.10357918	1.3257047	5.140415	0.8976922	0.3116078	0.5583806	-0.04082232	0.013036978	0.4235358
Hogmansbod	PaFTL2	6.5-h dark	2	18.28547048	1.3125175	5.113656	0.9022274	0.3183256	0.5550212	-0.02630423	0.080531553	0.201715
Jamtland	PaFTL2	6.5-h dark	2	17.29425475	1.3877441	5.266614	0.8762897	0.2797363	0.574224	-0.109184	0.006354538	0.495844
Norrland	PaFTL2	6.5-h dark	2	17.64706142	1.3599998	5.210176	0.8858511	0.2939395	0.5671448	-0.07861946	0.010067361	0.4505291
Saleby	PaFTL2	8-h dark	2	24.77478686	0.9687268	4.27484	0.6838827	0.07255829	1.0828521	0.1768407	0.003396988	5.52E-01
Istevallen	PaFTL2	8-h dark	2	24.77478686	0.9687268	6.157942	0.4909119	0.08499913	0.6476207	0.1405572	2.92438E-05	8.22E-01
Hogmansbod	PaFTL2	8-h dark	2	24.77478686	0.9687268	6.171123	0.4840324	0.08173737	0.6302373	0.1427557	0.001806262	6.03E-01
Jamtland	PaFTL2	8-h dark	2	24.77478686	0.9687268	6.706634	0.3957059	0.06491306	0.4196627	0.1473204	0.170061665	0.09732673
Norrland	PaFTL2	8-h dark	2	24.77478686	0.9687268	6.622679	0.4225862	0.07545146	0.4864659	0.1408149	0.010812493	0.44318458
Saleby	PaGI	6.5-h dark	1	31.50571317	0.7617666	0.2253251	-1.2264843	0.4083669	0	0	6.09E-04	0.9499638
Istevallen	PaGI	6.5-h dark	1	29.56801864	0.8116878	0.2930902	-0.9799559	0.3509365	0	0	0.001747929	0.9156402
Hogmansbod	PaGI	6.5-h dark	1	32.45252669	0.7395418	0.1951597	-1.3362269	0.4339268	0	0	0.002272174	0.9039764

Jamtland	PaGI	6.5-h dark	1	32.48286427	0.7388511	0.1942244	-1.3396267	0.4347236	0	0	0.011108401	0.7911934
Norrland	PaGI	6.5-h dark	1	30.46918353	0.7876811	0.2605084	-1.0984848	0.3785437	0	0	0.039517145	0.6173051
Saleby	PaGI	8-h dark	1	24	1	0.5344578	-1.426126	0.1689911	0	0	0.00099348	9.36E-01
Istevallen	PaGI	8-h dark	1	24	1	0.6460503	-1.294352	0.157305	0	0	0.022429406	7.07E-01
Hogmansbod	PaGI	8-h dark	1	24	1	0.5815179	-1.366374	0.1630477	0	0	0.001191218	0.9302158
Jamtland	PaGI	8-h dark	1	24	1	1.0786341	-1.04714	0.1753259	0	0	0.003227804	0.8858474
Norrland	PaGI	8-h dark	1	24	1	0.8727394	-1.21923	0.1798153	0	0	0.008237298	0.8194167