Nucleotide polymorphism and phenotypic associations within and around the phytochrome B2 locus in European aspen (Populus tremula, Salicaceae)

Pär K. Ingvarsson*1, M. Victoria Garcia1, Virginia Luquez2, David Hall1, Stefan Jansson2

1Umeå Plant Science Centre, Department of Ecology and Environmental Science, Umeå University, SE-901 87 Umeå, Sweden
2Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, SE-901 87 Umeå, Sweden

Email: Pär K. Ingvarsson* - par.ingvarsson@emg.umu.se;

*Corresponding author
Abstract

We investigated the utility of association mapping to dissect the genetic basis of naturally occurring variation in bud phenology in European aspen (*Populus tremula*). With this aim, we surveyed nucleotide polymorphism in 13 fragments spanning an 80 kb region surrounding the *phytochromeB2* locus. Although polymorphism varies substantially across the *phyB* region, we detected no signs for deviations from neutral expectations. We also identified a total of 41 SNPs that were subsequently scored in a mapping population consisting of 120 trees. We identified two non-synonymous single nucleotide polymorphisms (SNPs) in the *phytochromeB2* gene that were independently associated with variation in the timing of bud set, and that explained between 1.5 and 5% of the observed phenotypic variation in bud set. Earlier studies have previously shown that the frequencies of both these SNPs vary clinically with latitude. Linkage disequilibrium across the region was low, suggesting that the SNPs we identified are strong candidates of being causally linked to variation in bud set in our mapping populations. One of the SNPs (T608N) one is located in the "hinge region", close to the chromophore binding site of the *phyB* protein. The other SNP (L1078P) is located in a region supposed to mediate downstream signaling from the *phyB* locus. The lack of population structure, combined with low levels of linkage disequilibrium, suggest that association mapping is a fruitful method for dissecting naturally occurring variation in *Populus tremula*. 

2
INTRODUCTION

A major goal of population and quantitative genetics is to identify the polymorphisms underlying phenotypic variation, particular in traits that are important for ecological adaptations (Feder and Mitchell-Olds, 2003; Stinchcombe and Hoekstra, 2007). While the accumulation of functional genomics data over the last decades has provided detailed information on the genetic basis of many traits in a number of model organisms, it remains largely unknown how many of these genes that contain genetic variation segregate in natural populations. Progress in elucidating the genetic basis of ecological adaptations have been slow, partly because many of the species for which detailed information on phenotypic variation in traits of ecological relevance is available, are lacking the genomic resources that allow genetic dissection of these traits. Furthermore, adaptive traits are often quantitative and are therefore unlikely to have a simple genetic basis. Nevertheless, by studying the genetic basis of ecologically important traits hopes have been raised that it should yield insights into the number and effect sizes of genes underlying ecological adaptations and the evolutionary forces that act to maintain variation in such traits (Feder and Mitchell-Olds, 2003; Stinchcombe and Hoekstra, 2007).

Many organisms, like the model species Drosophila and Arabidopsis, are suitable for "classical" genetic techniques, based on the development of segregating mapping populations (Liu, 1998). However, for many organisms (e.g. humans) these tools are not feasible and other methods have been developed to dissect the genetic basis of phenotypic traits. One such method is association mapping, where unrelated individuals that have undergone recombination over multiple generations are genotyped and used to connect genotype to phenotype (Risch, 2000).

Forest genetics is in many respects in a similar situation to human genetics, because even though segregating tree populations can in principle be constructed, the extended juvenile phases and irregular flowering of many tree species makes such experiments impractical as they may take decades to complete. However, tree species have many characteristics that make them suitable for association mapping; they are predominantly outcrossing and have large, relatively unstructured populations, resulting in high levels of nucleotide diversity and low linkage disequilibrium (LD) (Neale and Savolainen, 2004; González-Martínez et al., 2006). In addition, many tree species can easily be cloned, allowing for phenotyping with high precision and for replication in different environments. Incidentally, the first papers using association mapping in forest trees
have recently been published (THUMMA et al., 2005; GONZÁLEZ-MARTÍNEZ et al., 2007).

Forest trees often have very wide geographic distributions and are ecologically dominant
species in many ecosystems (GONZÁLEZ-MARTÍNEZ et al., 2006; BRUNNER et al., 2004).
Because of their perennial nature, most tree species are subjected to large seasonal variations in
temperature and have as a response evolved annual growth cycles that promote long-term survival
and growth (GONZÁLEZ-MARTÍNEZ et al., 2006). While growth cessation and dormancy are
critical to winter survival, dormancy also constrains growth by reducing the amount of time
during which growth can take place (GONZÁLEZ-MARTÍNEZ et al., 2006; HORVATH et al.,
2003). A correct tuning of phenology to changes in environmental conditions across a growing
season thus represents an important ecological and evolutionary tradeoff between survival and
growth in most forest trees (GONZÁLEZ-MARTÍNEZ et al., 2006; HORVATH et al.,
2003). Seasonal variation in photoperiod is an important environmental variable that many tree species
use as a cue to initiate and/or terminate growth or reproduction (HOWE et al., 2003;
GONZÁLEZ-MARTÍNEZ et al., 2006). This is clearly manifested in the adaptive response of trees
to the steep latitudinal gradient in the length of growing season that characterizes northern
environments (HOWE et al., 2003; GONZÁLEZ-MARTÍNEZ et al., 2006). Many perennial plants
also show latitudinal clines in important phenological traits, such as timing of germination,
dormancy or the onset of flowering (HOWE et al., 2003; GONZÁLEZ-MARTÍNEZ et al., 2006).
Such clinal variation is usually interpreted as strong evidence for a balance between spatially
variable selection and migration (BARTON, 1999; GONZÁLEZ-MARTÍNEZ et al., 2006).

In many perennial plants, seasonal control of phenology is regulated by genes in the
photoperiodic pathway (WAREING, 1956; MOURADOV et al., 2002; HORVATH et al., 2003;
HOWE et al., 2003). Phytochromes are signal-transducing photoreceptors that are key
components of the photoperiodic pathway in plants (SMITH, 2000) and that play an important
role in light perception in plants. Known phytochrome-regulated processes includes the daily
entraining of the circadian clock, de-etiolation, stem elongation and seasonal developmental
responses such as flowering and initiation or release of dormancy (SMITH, 2000). The
photosensory activity of the phytochrome protein stems from its ability to convert between a
biologically inactive form, Pr, absorbing light at red wavelengths and a biologically active form,
Pfr, absorbing light at far-red wavelengths (SMITH, 2000; QUAIL, 2002). The conversion between
the Pr and Pfr form involves conformational changes of the molecule that allows for changes in
signalling activity of the phytochrome protein (MALOOF et al., 2000). The ratio of red to far-red
light changes both over short (a single day) and long (over a season) time scales and
phytochromes can thus provide a plant with temporal signals that are used to synchronize
developmental changes, such as the initiation or release of dormancy, with changing
environmental conditions across a growing season (SMITH, 2000). Phytochromes are thus ideal
candidate genes for mediating ecologically important variation in the timing of developmental
processes such as dormancy or flowering (MALOOF et al., 2000). Incidentally, mutations in two
members of the phytochrome gene family, PHYA and PHYC have been shown to be responsible
for natural variation in light sensitivity, seedling growth and flowering time in Arabidopsis
thaliana (MALOOF et al., 2001; BALASUBRAMANIAN et al., 2006).

Several replicated QTL mapping experiments in Populus have shown that one phytochrome
gene, phytochrome B2 (phyB2), maps to a linkage group containing QTLs for both bud set and
bud flush. phyB2 is thus a strong candidate gene for controlling naturally occurring variation in
bud phenology in Populus. (FREWEN et al., 2000; CHEN et al., 2002). We have shown that
populations of European aspen (Populus tremula) collected across latitudinal gradient
representing growing seasons ranging from two to five months show little population structure at
neutral markers but nevertheless show strong adaptive population differentiation in photoperiod
sensitivity (LUQUEZ et al., 2007; HALL et al., 2007). We have also documented clinal variation
with latitude at several non-synonymous mutations at phyB2 (INGVARSSON et al., 2006). These
sites are spread over roughly 4 kb of the phyB2 gene (INGVARSSON et al., 2006) and are likely to
represent independent clines, as linkage disequilibrium decline to negligible levels in less than
500 bp in P. tremula (INGVARSSON, 2005). In this paper we use association mapping to further
study the role of phyB2 in the mediating natural variation in photoperiodic control of dormancy
initiation and release in P. tremula. We show that two amino acid substitutions are associated with
natural variation in bud set in P. tremula.

MATERIAL AND METHODS

Plant material and phenotypic scoring  All data described in this paper were generated
using trees from the Swedish Aspen Collection (the SwAsp collection) that have been described
in detail elsewhere (INGVARSSON et al., 2006; HALL et al., 2007; LUQUEZ et al., 2007). This collection was established from ten *P. tremula* clones collected from each of twelve different sites throughout Sweden in 2003. All trees were clonally replicated (four rametes per clone) and planted in at two common garden sites in 2004 (Sävar, 63°N and Ekebo, 56°N). A total of 480 ramets were planted at each common garden site. All data presented here are based on means from four rametes per clone at each site. During the spring, summer and autumn of 2005 and 2006 we measured bud flush and bud set traits at the two common garden sites. Bud flush (BF) was scored every two days from the flush of the first tree in the spring until all trees had flushed. A tree was considered to have flushed when the first fully unfolded leaf was observed (FREWEN et al., 2000). Bud set (BS) was scored twice a week starting in mid-July and was continued until trees had set terminal buds (FREWEN et al., 2000). In 2006 we also scored bud flush and bud set under greenhouse conditions. At least two rametes of each clone were kept in either of two greenhouses at the Swedish University of Agricultural Sciences, Umeå under ambient photoperiod. Bud flush and bud set were scored in the same manner as in the field planted trees. We have excluded data for bud set from Sävar in 2006 in all of our analyzes, as a severe drought caused most of the trees to set buds prematurely, in the middle of the season.

### SNP discovery and genotyping
Total genomic DNA was extracted from frozen leaf tissue using the DNeasy plant mini prep kit (Qiagen Inc. Valencia, CA). Primers to amplify 12 fragments of roughly 800 bp surrounding the *Populus phyB2* gene were designed based on the publicly available genome sequence of *P. trichocarpa* ([http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html](http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html)). The primer combinations were tested to ensure amplification and if the PCR reactions failed new primers were designed. An effort was made to have a roughly equal spacing between fragments, but that was sometimes not possible because of an apparent lack of conservation of intergenic regions between *P. trichocarpa* and *P. tremula*. All primer sequences used are presented in Supplementary Table S1. Fragments were PCR amplified from a total of 12 individuals from the SwAsp collection. PCR products were cloned into the pCR2.1 vector using a TA-cloning kit from Invitrogen (Carlsbad, CA) and fragments were sequenced either on an ABI377 automated sequencer or a Beckman CEQ 2000 capillary sequencer at the Umeå Plant Science Centre sequencing facility. As *P. tremula* is highly heterozygous (INGVARSSON, 2005), five or more
clones from three pooled PCR reactions were sequenced in an attempt to identify the two haplotypes present within an individual and to control for Taq polymerase errors. Sequences were verified manually and contigs were assembled using the computer program Sequencer v 4.0. Multiple sequence alignments were made using Clustal W (THOMPSON et al., 1994) and adjusted manually using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All sequences described in this paper have been deposited in the EMBL database (accession numbers pending). SNPs were identified from the sequenced fragments and were scored either by developing cleaved amplified polymorphism sequence markers (CAPS), by single base pair primer extension on a Beckman CEQ 8000 capillary sequencer or by complete sequencing of short fragments amplified from genomic DNA (INGVARSSON et al., 2006).

**Statistical analyses** Estimates of nucleotide diversity per site from the total number of segregating sites (θ), average pairwise heterozygosity (π) and Tajima’s D (TAJIMA, 1989) were obtained from the sequence fragments using a computer program written in C++ based on the publicly available C++ class library libsequence (THORNTON, 2003). Test for Hardy-Weinberg equilibrium were performed for each SNP using the genetics (WARNES and LEISCH, 2006) package from the statistical package R (R DEVELOPMENT CORE TEAM, 2007). This package was also used to test for pairwise linkage disequilibrium between individual SNPs using a maximum likelihood approach since the genotype data is unphased and individual haplotypes cannot be distinguished. We also tested for climatic variation in SNP frequencies by regressing population allele frequencies on latitude of origin.

In our association analyses, we preferred single marker tests over haplotype-based tests for several reasons. First, there are uncertainties in haplotype determination from diploid, unphased SNP data. Second, LD between markers was fairly low, yielding a large number of potential haplotypes with even a few SNPs, and haplotype-based analyses are known to rapidly loose power with increasing numbers of haplotypes. Finally, single-marker tests have equal or even higher power than haplotype tests if LD is low and/or there is a high probability that causal SNPs have been typed (LONG and LANGLEY, 1999).

Single marker associations were tested using a model assuming additive effects of alleles within a marker locus. To control for possible spurious associations caused by genetic structuring of the sample, we used the mixed-model method of YU et al. (2006) that allows for both
population structure and more diffuse familial structure within the sample. In matrix form this model is given by

\[ z = X\beta + Qv + Zu + e \]  

(1)

where \( z \) is a vector of phenotypic observations, \( \beta \) is a vector of SNP effects, \( v \) is a vector of population effects, \( u \) is a vector of polygenic background effects and \( e \) is a vector of residual effects.

Population structure and pairwise kinship coefficients were estimated using 26 putatively neutral SSR markers and 39 SNPs from four different genes (\( \text{col}2b, \text{GA}20\text{ox}1, \text{hypO}312 \) and \( \text{abi}1b \)) (HALL et al., 2007). Population structure was estimated in two different ways. We either used the program Structure (PRITCHARD et al., 2000) to estimate population membership for each individual for a number of different subpopulations (\( K=2 \) to 5) or used the principle-component (PCA) method of PRICE et al. (2006) to infer population structure. The effects of population structure, estimated using either Structure or using PCA, were summarized by the \( Q \) matrix in Equation (1). Both methods yielded similar results, and we therefore only present data based on Structure. Similarly, a matrix of pairwise kinship coefficients, \( K \), was calculated according to RITLAND (1996) using the software package SpaGeDi (HARDY and Vekemans, 2002) and was used to specify the variance of the random (clone) effects according to \( V'(u) = 2KV_g \). Finally, the variance of the residuals was assumed to be \( V'(e) = RV_R \), where \( R \) is a matrix with zeros for all off-diagonal elements and the reciprocal of the number of observations along the diagonal. \( V_g \) and \( V_R \) are the genetic and residual variances for the trait in question.

We combined data from the different sites and years by calculating best linear unbiased predictors (BLUPS) for all genotypes in the association population by fitting the model

\[ z_{ijkl} = \mu + b_i + \alpha_j + \gamma_k + e_{ijkl} \]  

(2)

where \( z_{ijkl} \) is the phenotype of the \( l \)th individual in the \( k \)th block from the \( j \)th clone from the \( i \)th population. In Equation (2) \( \mu \) denotes the grand mean and \( e_{ijkl} \) is the residual error term. The clone (\( \beta_j \)) and residual term (\( e_{ijkl} \)) were modeled as random effects, whereas site/year (\( b_i \)) and block (\( \gamma_k \)) were treated as a fixed effects. Equation (2) was fitted to the data using restricted maximum likelihood techniques and BLUPS were calculated using the \texttt{lmer} function in \texttt{R}. The BLUPS were used as the dependent trait in the association analyses. We implemented the
mixed-model analysis (YU et al., 2006) using the kinship library (ATKINSON and THERNEAU, 2007) in R, and fitted the models using maximum likelihood methods. To control for multiple testing we used method of STOREY and Tibshirani (2003) to control the false discovery rate (FDR), as implemented in the qvalue package in R.

Data-perturbation simulations We used the data-perturbation method described in YU et al. (2006) to create new data sets that were analyzed using the same methods as the observed phenotypic data. Briefly, a single SNP is randomly chosen and assigned a phenotypic effect which is added to the original data. By applying this to the original data, the complex correlation structure of the data is preserved. Phenotypic effects of SNPs were chosen in the range of 0.0 to 1.0 times the phenotypic standard deviation. The proportion of phenotypic variation explained by the causal SNP was estimated by regressing phenotype on SNP genotype (ZHAO et al., 2007). We scored power across the simulated data sets for three different $\alpha$-values, 0.05, 0.01, and 0.001. We also estimated the distribution of observed allelic effects conditional the “true” allelic effect and on observing a significant effect at a SNP at a given $\alpha$-value (ALLISON et al., 2002). For each replicate simulation where we obtained a significant result (at $p < 0.001$), we scored the true and estimated phenotypic effect of the SNP. This simulated distribution was then used to calculate corrected estimated of allelic effects at the T608N and L1078P SNPs, using the method of moments approach outlined in ALLISON et al. (2002).

RESULTS

Molecular population genetics and SNP identification We surveyed nucleotide polymorphism in a sample of 12 short (c. 800 bp) fragments spanning roughly 80 kb surrounding the phyB2 gene (Figure 1, Supplementary Table S1). Nucleotide diversity ($\pi$) vary substantially across the phyB2 region, there is an almost four-fold variation in polymorphism at silent sites ranging from $\pi_S = 7.4 \times 10^{-3}$ at phyLD16 to $\pi_S = 31.5 \times 10^{-3}$ at phyLD18 (average $\pi_S = 18.1 \times 10^{-3}$) (Figure 1). There are also large differences in the frequency spectrum of segregating mutations between the different fragments, with some fragments having an excess of mutations at low frequency (a negative Tajima’s D) while others have an excess of mutations at intermediate frequencies (positive Tajima’s D, Figure 1). Nevertheless, despite the large variation
seen in polymorphism and frequency spectra across the 12 fragments, there are no indications for a deviation from neutral expectations across the phyB2 region, as indicated by a non-significant HKA-test (Hudson et al., 1987) ($\chi^2 = 5.41$, $df = 11$, $p = 0.91$). This suggests that the entire 80 kb region surrounding the phyB2 locus is evolving according to neutral expectations and confirms results from a previous study (Ingvarsson et al., 2006), which showed little evidence for non-neutral evolution within the phyB2 gene itself. We identified 29 SNPs that had a minor allele frequency greater than 0.1 in the fragments surrounding the phyB2 gene (an average of 2.4 SNPs per fragment). We scored an additional 5 SNPs from the phyB2 gene and added these to the 9 SNPs we had previously scored in the SwAsp collection (Ingvarsson et al., 2006). A total of 42 SNPs were therefore scored in all individuals from the SwAsp collection.

**Population structure and allele frequency clines in the phyB2 region** Neutral markers show little evidence for populations structuring in the SwAsp collection; global genetic differentiation, measured as $F_{ST}$, from 26 SSR loci is 0.015 (Hall et al., 2007). Although this estimate of $F_{ST}$ is significantly greater than zero (Hall et al., 2007), such low population differentiation suggest that *P. tremula* is essentially panmictic across the region from which the trees were originally sampled. This is also demonstrated by our attempts to infer population structure from the SSR marker data using the program Structure (Pritchard et al., 2000). Our analyses showed patterns typical of unstructured populations, such as a roughly equal allocation of individuals to the inferred populations and with most individuals showing evidence for admixture. Also, the method outlined in Evanno et al. (2005) identified only a single population, demonstrating the lack of population structuring at neutral markers in the SwAsp collection. There was also little structuring within populations in terms of relatedness among the sampled trees, with the mean pairwise relatedness among trees being 0.01 and statistically indistinguishable from zero.

In an earlier study of polymorphisms in the phyB2 gene, we demonstrated that four out of nine SNPs scored showed significant clinal variation (Ingvarsson et al., 2006). That study included two populations from Southern Europe and it is possible that the strong clinal variation we documented at phyB2 was partly caused by the inclusion of these outlier populations. We therefore tested for clinal variation in population frequencies at each of the 42 SNPs. We detected significant ($p < 0.05$) clinal variation at eight SNPs, three of which remain significant after
multiple-test correction. These three SNPs are all located within the phyB2 gene. The large number of SNPs in the phyB2 gene showing evidence for clinal variation are unlikely to be a result of chance. Clinal variation is not a common observation in *P. tremula*; 39 SNPs from four other candidate genes for bud phenology (GA20ox1, col2B, ABI1B and hypO312), yielded no evidence for clinal variation (HALL et al., 2007) and the same is true for several other genes we have surveyed (unpubl. data). The pattern of clinal variation that we observe at SNPs in the phyB2 region is therefore likely to be related to adaptive differentiation to photoperiod.

**Association analyses** As suggested by the molecular population genetic analyses, linkage disequilibrium (LD) varied across the phyB2 region, but overall the effects of LD were relatively low. A total of 110 out of 861 pairwise comparisons (12.8%) between SNPs showed evidence for significant LD after multiple-test corrections, but there was no clear physical clustering of sites in LD (Figure 2). Sites in close physical proximity often showed negligible levels of LD while sites separated by several thousand kb sometimes showed a signal of strong LD (Figure 2). However, most sites showing long-range LD are mutations occurring in relatively low frequencies (Supplementary Table S2).

We scored bud flush and bud set in two different common gardens, one in southern Sweden (Ekebo) and one in northern Sweden (Sävar) (LUQUEZ et al., 2007), using four rametes per clone at each garden. We also scored bud flush and bud set in a greenhouse in 2006, using two rametes per clone. In addition we had phenotypic data from two years from Ekebo, resulting in a total of four phenotypic data sets. We combined these different data sets and calculated best unbiased linear predictors (BLUPs) for the individuals in our association mapping population for both bud set and bud flush. These BLUPs were then used as traits in our association analyses.

We did not detect any effects of population structure in our association test, as the effect of population structure, estimated either using Structure (PRITCHARD et al., 2000) or using PCA (PRICE et al., 2006), was negligible. The random kinship effect was highly significant for bud set where it explained about 7% of the observed variation, where as for bud flush including the kinship matrix did not explain any additional variation. We also calculated kinship using the allele sharing method described by ZHAO et al. (2007). This measure, however, explained somewhat less variation (5%) than the classical kinship estimate (RITLAND, 1996), so we only present results based on the latter.
After correcting for multiple testing, two SNPs showed significant associations with bud set (Table 1, Supplementary Table S2). These two SNPs are non-synonymous mutations (T608N and L1078P (INGVARSSON et al., 2006)) located within the phyB2 gene itself. T608N are located in exon 1 in the vicinity of the chromophore binding site and L1078P is located in exon 4, in a region of the phyB2 protein that is hypothesized to be involved in signalling transduction (SMITH, 2000). To evaluate whether these SNPs represents mutations with independent effects on bud set, we fit a model where both SNPs were included as explanatory variables. This tests for the effect of a SNP while statistically controlling for variation at the other possible causal SNP. This analysis shows that as both SNP are independently associated with bud set. This is expected, since there is no evidence for any association between T608N and L1078P, with LD being low and non-significant ($r^2 = 0.044, p > 0.5$, Figure 2). There is thus strong evidence for the T608N and L1078P mutations having independent effects on bud set in P. tremula and there is no evidence that these associations are caused by linkage disequilibrium between the two mutations.

We also tested all SNPs for associations with bud flush to determine whether the putative causal polymorphisms we identified above were specific to bud set or whether they are generally involved in regulating bud phenology. Bud flush is known to be strongly influenced by temperature in P. tremula. Temperature, on the other hand, has only minor influence on bud set (LUQUEZ et al., 2007), so pleiotropic effects of mutations in phyB2 are perhaps unlikely. Nevertheless, QTLs for bud flush has been demonstrated to co-locate with phyB2 in multiple independent mapping populations (FREWEN et al., 2000; CHEN et al., 2002). We did not, however, detect any SNP-phenotype associations with bud flush in any of our experiments. This is intriguing since the phyB2-associated QTL for bud flush explain roughly 10% of the segregating variation in bud flush, which is about 50% greater than the bud set associated QTL that was also co-located with phyB2 (FREWEN et al., 2000). However, this could be explained by the fact that the mapping populations were based on interspecific crosses (FREWEN et al., 2000; CHEN et al., 2002) and there either is more variation segregating in these populations than in natural populations or that that genetic variation segregating in the mapping populations represents variation that is normally fixed between the two species and that hence would not be found segregating within a species.
Effect sizes of SNPs associated with bud set  The two non-synonymous SNPs in \( \text{phyB} \) that we found to be associated with bud set each explain approximately 8% of the phenotypic variation in bud set in our association population (Table 1, see also Supplementary Table S2). The additive effects associated with the two SNPs corresponds to shifts in bud set with 4.4 and 5.2 days, respectively, which corresponds to 42% and 49% of the phenotypic standard deviation in bud set (Figure 3). We have, however, every reason to believe that these values are overestimating the true effects of the two mutations. Estimating the phenotypic effects of mutations from the same data that were used to establish an association leads to an ascertainment bias where the SNPs showing significant associations are also the ones that tend to be associated with the strongest phenotypic differences among genotypes (sometimes termed the “Beavis effect” (XU, 2003) or the “winner’s curse” (ZÖLLNER and PRITCHARD, 2007)).

In attempt to evaluate the degree by which these estimates are upwardly biased, we performed numerical simulations to investigate the power of our association analysis. Simulations show that our power to detect associations is generally quite low, even if the causal mutation is included in the sample (Supplementary Figure S1). Only when the phenotypic variation explained by a mutation is quite large (∼10%) does power reach reasonable levels. In situations where the causal mutation is not included in the sample of SNPs analyzed power uniformly low, consistent with the low levels of LD observed in \( \text{P. tremula} \) (INGVARSSON, 2005). The degree by which the additive effect of an allele is overestimated is a function of the power of the study, such that with lower power, allelic effects will be more upwardly biased (GÖRING et al., 2001; ALLISON et al., 2002; XU, 2003; ZÖLLNER and PRITCHARD, 2007). This effect is apparent in our simulations, where mutations with small effects can be overestimated by more than a factor of two (Supplementary Figure S2).

ALLISON et al. (2002) suggested an \textit{ad hoc} method for correcting the ascertainment bias introduced when estimating allelic effects at loci from the same data set that were used to identify the loci. The method of ALLISON et al. (2002), was originally developed for QTL mapping studies, but it is easily adapted to association mapping studies. It is a method of moments approach that builds upon estimating the (truncated) distribution of allelic effects, \( \hat{a} \), given the underlying distribution of (true) allelic effects \( a \) and a specified significance cut-off level \( \alpha \). Once this distribution, \( E(\hat{a}|a, p < \alpha) \), has been obtained it can be equated with the observed allelic
effects $a_{\text{obs}}$ to get an estimate of the underlying allelic effect for a given mutation.

Although this distribution could be analytically derived in certain cases, Allison et al. (2002) suggested using numerical simulation to approximate the distribution. Applying this method to our association mapping data, suggest that the naive estimates of the effects of the two mutations are upwardly biased by factors of 2.5 and 1.4, respectively. However, taking this reduction in effect size into account, the T608N and L1078P mutations still explain 1.4% and 5.9% of the variation in bud set.

DISCUSSION

The low LD seen in most forest trees suggest that fine scale mapping should be possible. However, low LD currently makes full genome scans very inefficient, since several millions of markers would have to be scored to have reasonable chances to find significant associations. It has therefore been suggested that a candidate gene approach, where fine-scale mapping is applied to a selected set of candidate genes, identified from, for instance, QTL mapping experiments or from functional genomic studies in model species like Arabidopsis, could be a promising approach to dissect quantitative traits in forest trees (Neale and Savolainen, 2004). In this paper we have used a candidate gene approach in P. tremula to study the genetic basis of bud phenology, an ecologically important trait in long-lived tree species.

We found that two mutations (T608N and L1078P) in the photoreceptor gene phyB2 are independently associated with naturally occurring variation in bud set in P. tremula (Table 1 and Supplementary Table S2). The two mutations also show parallel clines in allele frequencies (Ingvarsson et al., 2006). Despite this, these SNPs does not show stronger genetic differentiation than randomly chosen microsatellite markers or SNPs (Hall et al., 2007). This result fits theoretical expectations, which show that genetic differentiation at QTLs is better approximated by genetic differentiation at neutral loci than by genetic differentiation in the quantitative traits themselves (Latta, 1998; Le Corre and Kremer, 2003). In the presence of high levels of gene flow, spatially variable selection generates covariances between individual QTNs (i.e. linkage disequilibrium) that reinforce the total phenotypic effect of the QTLs (Latta, 1998; Le Corre and Kremer, 2003). When these covariances are positive, they reinforce the total effect of the QTLs and result in large population differences in the quantitative
traits, despite small changes in frequencies of the underlying QTLs (Latta, 1998; Le Corre and Kremer, 2003). These covariances reflect an among-population component of LD that develops in the face of diversifying selection and is thus distinct from the low intra-population LD that is generally observed in Populus (Ingvarsson, 2005). Taken together, our results suggest that the two phyB2 mutations we observe to be associated with bud represent independent evolutionary “solutions” to the problem of adapting bud set to seasonal differences in the length of the growing season.

Given that we found two SNPs showing significant association with bud set, a trait of critical ecological importance for perennial trees, it may seem somewhat peculiar that we did not detect any evidence of natural selection acting on phyB2. All the population surveyed must have been established within the last 10 kyr, following the last glaciation, so the latitudinal cline we observe in bud set must have been established in relatively short time. This, combine with the fact that the cline is still actively maintained today, suggest that natural selection acting on these mutations must be quite strong. However, recombination rates appear to be high in P. tremula, as these is very little LD in this species (Ingvarsson, 2005). This is clearly important, since the region affected by selection is a function of both the strength of selection acting on a beneficial mutation and the recombination rate in the region surrounding the mutation (Kim and Stephan, 2002).

However, it is possible that these SNPs are quite old and that they have been maintained in the species over evolutionary time. If so, these mutations may have persisted at appreciable frequencies in glacial refugia populations. In this case, natural selection acting on these SNPs during post-glacial colonization would leave even less of a trace in the polymorphism data in and surrounding phyB2 (Przeworski et al., 2005). This is clearly something that is worth studying further.

It is interesting to note that it likely would not be possible to identify these mutations using traditional QTL mapping, without prior knowledge of their existence. Rather, a QTL mapping experiment would confound the two mutations and estimate their combined effect. For phyB2, this would not preclude the identification of a QTL in the region, as the two mutations appear to act additively to influence bud set. However, if a QTL is composed of causal mutations having opposing effects, the probability of actually detecting the QTL in a mapping populations could be drastically reduced (Kroymann and Mitchell-Olds, 2005). One thing worth pointing out
here is that clinal variation at a SNP does not guarantee an association with bud set, a trait that is also showing clinal variation (Ingvarsson et al., 2006; Hall et al., 2007; Luquez et al., 2007). This is evident from the fact that we did not detect significant associations with bud set for all SNPs showing clinal variation; two SNPs in phyB2 where we have showed significant clinal variation (L789M and Int3) (Ingvarsson et al., 2006) are not statistically associated with bud set.

The QTL mapping experiments which initially demonstrated the co-location of phyB2 and a bud set QTL (Frewen et al., 2000; Chen et al., 2002), found a minimum of 4 QTLs contributing to bud set. These mapping populations were based on interspecific crosses, however, and it is not clear whether this affects the estimation of both the number of QTLs that contribute to a trait and the magnitude of the effects of these QTLs. Nevertheless, the QTL that co-located with phyB2 explained 6.8% of the variation in bud set in the mapping population and none of the four QTLs detected explained more than 12.2% (Frewen et al., 2000). The amount of variation explained by a QTL depends both the quality of the phenotypic data and the size of the mapping populations used when estimating effect sizes (Xu, 2003). It is therefore hard to directly compare effect sizes between experiments. The naive estimate of the phenotypic variation explained by the two mutation we identified in this study is around 8%. Effect sizes of QTLs are known, however, to be upwardly biased when they are estimated from small mapping populations, and this effect is more severe for mutations of small effect (Göring et al., 2001; Allison et al., 2002; Xu, 2003). We employed an ad hoc method (Allison et al., 2002) in an effort to obtain less biased estimates of the effects of the T608N and L1078P mutations. By taking the ascertainment bias into account, the effects of the T608N and L1078P SNPs are reduced by a factor of 1.4 and 2.5, respectively. Nevertheless, even with their reduced effect sizes, the T608N and L1078P SNPs each explain a sizable fraction of the variation seen in bud set (1.4 and 5.9%). These two mutations thus appear to be important determinants of naturally occurring phenotypic variation in bud set in P. tremula. One thing worth pointing out is that the method of moments approach critically depends on a correct specification of the underlying genetic model (Allison et al., 2002). We have used a simple additive model in all our association analyzes, because we found little evidence for dominance at any of the SNPs, and this model was also used in our data-perturbation simulations. Another drawback of the method of moment approach is that it
only provides point estimates of the allelic effects and that there is now obvious way to place confidence bounds on these estimates.

At present, we can only speculate about the possible functions of the two mutations. The phytochrome protein occurs as a homodimer in solution and is made up of two structural domains with a chromophore-bearing N-terminal half and a C-terminal half involved in determining regulatory specificity (Smith, 2000). The T608N mutation is located in the hinge region, at the border between the two structural domains of the protein, close to regions that are involved in dimerization and in mediating conformational changes between the Pr and Pfr form of the phyB2 protein (Quail, 2002; Chen et al., 2004). It is thus possible that mutations in this region could affect either the stability of the homodimer or the rate of conformational changes between the Pr and Pfr forms. Such changes could possibly affect phytochrome sensitivity to either red and/or far-red light. This is interesting, since not only day length, but also the spectral composition of light (‘light quality’), varies latitudinally and studies have documented clinal responses to light quality in several tree species ((Clapham et al., 1998) and references therein). The L1078P mutation, on the other hand, is located in the extreme C-terminal part of the phyB2 protein. This region of the protein is poorly characterized but have sequence similarities with prokaryotic histidine kinases. Incidentally, serine/threonine kinase activity is present in phytochromes, but it is not clear whether and how this is involved in mediating phytochrome signalling (Quail, 2002; Chen et al., 2004). It is thus possible that the L1078P mutation might affect signalling to downstream components in the photoperiodic pathway. Further studies of the two phyB2 mutations are clearly needed, both to validate their association with natural variation in bud set in P. tremula but also to gain further insights into their possible functional significance. The possible interplay between the three phytochromes present in Populus, and potentially other photoreceptors, allows for very intricate regulation of light-regulated development, and will require both forward genetics approaches - like this study - and reverse genetics experiments using transgenic plants, to fully elucidate. Also, although we found significant associations in a populations consisting of slightly more than a hundred trees, larger mapping populations are clearly needed to get less biased estimates of the true effect sizes of the QTNs identified and to have reasonable power to identify mutations with smaller phenotypic effects. We are therefore in the process of extending our experimental population to over 400 genotypes.
By identifying genes underlying ecologically important traits it should be able to address many important questions regarding the genetic architecture of quantitative variation. How common is it that a single QTL is composed of multiple, possibly linked causal polymorphisms? Furthermore, are adaptive mutations derived from standing genetic variation or do they represent newly arisen mutations, and if so, how long have these mutations been maintained within the species? Also, are parallel adaptations in different species due to mutation in the same set of genes? Future studies on the genetic basis of ecological adaptations in *Populus*, and in other closely related species, should begin to shed some light on this.

ACKNOWLEDGEMENTS

This study has been funded by grants from the Swedish Research Council (VR), the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas), Kempestiftelserna, the Swedish Foundation for Strategic Research (SSF) and the Research School in Forest Genetics and Breeding to PKI or SJ.

LITERATURE CITED


Xu, S., 2003 The theoretical basis of the Beavis effect. Genetics 165.
Figure 1: Polymorphism and Tajima’s D across the *phyB2* region. Dashed vertical lines gives the 95% confidence interval for the estimates of $\pi$. The boxes below the two figures represents genes surrounding the *phyB2* gene (black). Note that boxes represent entire genes and that introns are not displayed.
Figure 2: Linkage disequilibrium and phenotypic associations at the 42 SNPs. Strength of pairwise LD between SNPs are indicated color coding. The physical location of the SNPs in the genes surrounding the *phyB2* gene are indicated with dashed lines. As in Figure 1, filled boxes represent entire genes.
Figure 3: Genotypic effects (± SE) of phyB2 SNPs A) T608N and B) L1078P on bud set under the different experimental settings. The effect is displayed as a deviation from the mean time to bud set in each experiment.
Table 1: Associations between two non-synonymous SNPs and bud set in *P. tremula*

<table>
<thead>
<tr>
<th>Factor</th>
<th>SNP</th>
<th>2ΔL</th>
<th>Effect</th>
<th>p-value</th>
<th>FDR q-value</th>
<th>R²(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bud set</td>
<td>Kinship (K)</td>
<td>8.977</td>
<td>4.43</td>
<td>2.7 × 10⁻³</td>
<td>0.977</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>Population structure (Q/P)</td>
<td>0.002</td>
<td>1.81</td>
<td>9.8 × 10⁻⁴</td>
<td>0.033</td>
<td>9.0</td>
</tr>
<tr>
<td>SNP</td>
<td>T608N</td>
<td>10.857</td>
<td>5.19</td>
<td>1.6 × 10⁻³</td>
<td>0.033</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>corrected²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNP</td>
<td>L1078P</td>
<td>9.977</td>
<td>5.19</td>
<td>1.6 × 10⁻³</td>
<td>0.033</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>corrected²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bud flush</td>
<td>Kinship (K)</td>
<td>0.000</td>
<td>0.99</td>
<td>0.24</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Population structure (Q/P)</td>
<td>1.398</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹False discovery rate, ²SNP effects corrected using method of moments approach (ALLISON *et al.*, 2002)
Figure S1: Average adjusted power to detect mutations of a given phenotypic effect for the two traits studies. Three different significance levels were used, $p = 0.05$ (solid line), $p = 0.01$ (dashed line) and $p = 0.01$ (dotted line).
Figure S2: The distribution of simulated additive effects given the true additive effect. Black circles denote the simulated effect of mutations significant at $p < 0.001$. White circles are the simulated effects of all mutations, regardless of significance. Values in parentheses under the x-axis is the power to detect a significant effect at $p < 0.001$ for a given effect size.
Table S1: Summary statistics for sequenced fragments surrounding the *phyB2* gene
Table S2: Frequencies and phenotypic associations with bud set and bud flush for all 42 SNPs scored.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Site</th>
<th>Frequency</th>
<th>$2\Delta_L$</th>
<th>p-value</th>
<th>q-value</th>
<th>$2\Delta_L$</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD12</td>
<td>277</td>
<td>0.270</td>
<td>1.86</td>
<td>0.1726</td>
<td>0.4653</td>
<td>0.03</td>
<td>0.8682</td>
<td>0.9885</td>
</tr>
<tr>
<td>LD12</td>
<td>498</td>
<td>0.266</td>
<td>2.85</td>
<td>0.0911</td>
<td>0.4653</td>
<td>0.57</td>
<td>0.4516</td>
<td>0.8243</td>
</tr>
<tr>
<td>LD11</td>
<td>136</td>
<td>0.561</td>
<td>0.42</td>
<td>0.5192</td>
<td>0.8076</td>
<td>0.00</td>
<td>0.9647</td>
<td>0.9921</td>
</tr>
<tr>
<td>LD11</td>
<td>561</td>
<td>0.645</td>
<td>0.62</td>
<td>0.4327</td>
<td>0.8076</td>
<td>0.03</td>
<td>0.8642</td>
<td>0.9885</td>
</tr>
<tr>
<td>LD11</td>
<td>767</td>
<td>0.200</td>
<td>0.00</td>
<td>0.9818</td>
<td>0.9958</td>
<td>0.83</td>
<td>0.3629</td>
<td>0.8023</td>
</tr>
<tr>
<td>LD8</td>
<td>175</td>
<td>0.149</td>
<td>0.69</td>
<td>0.4065</td>
<td>0.8076</td>
<td>0.63</td>
<td>0.4261</td>
<td>0.8243</td>
</tr>
<tr>
<td>LD8</td>
<td>399</td>
<td>0.478</td>
<td>0.44</td>
<td>0.5069</td>
<td>0.8076</td>
<td>0.01</td>
<td>0.9179</td>
<td>0.9885</td>
</tr>
<tr>
<td>LD8</td>
<td>275</td>
<td>0.155</td>
<td>0.19</td>
<td>0.6609</td>
<td>0.8675</td>
<td>0.86</td>
<td>0.3533</td>
<td>0.8023</td>
</tr>
<tr>
<td>LD7</td>
<td>51</td>
<td>0.339</td>
<td>0.23</td>
<td>0.6351</td>
<td>0.8604</td>
<td>0.85</td>
<td>0.3568</td>
<td>0.8023</td>
</tr>
<tr>
<td>LD7</td>
<td>400</td>
<td>0.631</td>
<td>1.73</td>
<td>0.1883</td>
<td>0.4653</td>
<td>0.40</td>
<td>0.5262</td>
<td>0.8500</td>
</tr>
<tr>
<td>LD6</td>
<td>170</td>
<td>0.750</td>
<td>7.49</td>
<td>0.0062</td>
<td>0.0866</td>
<td>4.60</td>
<td>0.0320</td>
<td>0.4625</td>
</tr>
<tr>
<td>LD6</td>
<td>165</td>
<td>0.171</td>
<td>2.29</td>
<td>0.1300</td>
<td>0.4653</td>
<td>5.14</td>
<td>0.0234</td>
<td>0.4625</td>
</tr>
<tr>
<td>LD6</td>
<td>675</td>
<td>0.619</td>
<td>0.53</td>
<td>0.4657</td>
<td>0.8076</td>
<td>0.01</td>
<td>0.9134</td>
<td>0.9885</td>
</tr>
<tr>
<td>LD5</td>
<td>157</td>
<td>0.094</td>
<td>1.75</td>
<td>0.1857</td>
<td>0.4653</td>
<td>3.68</td>
<td>0.0551</td>
<td>0.4625</td>
</tr>
<tr>
<td>LD5</td>
<td>422</td>
<td>0.586</td>
<td>4.67</td>
<td>0.0306</td>
<td>0.3218</td>
<td>0.19</td>
<td>0.6610</td>
<td>0.9254</td>
</tr>
<tr>
<td>LD5</td>
<td>476</td>
<td>0.727</td>
<td>0.15</td>
<td>0.6969</td>
<td>0.8869</td>
<td>1.35</td>
<td>0.2455</td>
<td>0.6942</td>
</tr>
<tr>
<td>phyB2</td>
<td>49</td>
<td>0.928</td>
<td>3.69</td>
<td>0.0548</td>
<td>0.3834</td>
<td>0.11</td>
<td>0.7446</td>
<td>0.9773</td>
</tr>
<tr>
<td>phyB2</td>
<td>930</td>
<td>0.914</td>
<td>0.28</td>
<td>0.5985</td>
<td>0.8604</td>
<td>1.43</td>
<td>0.2321</td>
<td>0.6942</td>
</tr>
<tr>
<td>phyB2</td>
<td>1143</td>
<td>0.275</td>
<td>0.00</td>
<td>0.9550</td>
<td>0.9958</td>
<td>0.48</td>
<td>0.4907</td>
<td>0.8243</td>
</tr>
<tr>
<td>phyB2</td>
<td>1152</td>
<td>0.081</td>
<td>2.14</td>
<td>0.1437</td>
<td>0.4653</td>
<td>1.65</td>
<td>0.1993</td>
<td>0.6942</td>
</tr>
<tr>
<td>phyB2</td>
<td>1179</td>
<td>0.618</td>
<td>0.63</td>
<td>0.4257</td>
<td>0.8076</td>
<td>0.72</td>
<td>0.3949</td>
<td>0.8243</td>
</tr>
<tr>
<td>phyB2</td>
<td>1823</td>
<td>0.441</td>
<td>10.86</td>
<td>0.0010</td>
<td>0.0333</td>
<td>2.06</td>
<td>0.1507</td>
<td>0.6776</td>
</tr>
<tr>
<td>phyB2</td>
<td>1935</td>
<td>0.410</td>
<td>0.28</td>
<td>0.5941</td>
<td>0.8604</td>
<td>0.02</td>
<td>0.8910</td>
<td>0.9885</td>
</tr>
<tr>
<td>phyB2</td>
<td>2365</td>
<td>0.205</td>
<td>1.11</td>
<td>0.2912</td>
<td>0.6795</td>
<td>0.32</td>
<td>0.5714</td>
<td>0.8889</td>
</tr>
<tr>
<td>phyB2</td>
<td>5634</td>
<td>0.387</td>
<td>1.99</td>
<td>0.1579</td>
<td>0.4653</td>
<td>0.56</td>
<td>0.4526</td>
<td>0.8243</td>
</tr>
<tr>
<td>phyB2</td>
<td>6030</td>
<td>0.723</td>
<td>9.98</td>
<td>0.0016</td>
<td>0.0333</td>
<td>2.73</td>
<td>0.0984</td>
<td>0.5723</td>
</tr>
<tr>
<td>phyB2</td>
<td>6062</td>
<td>0.875</td>
<td>1.99</td>
<td>0.1584</td>
<td>0.4653</td>
<td>3.94</td>
<td>0.0473</td>
<td>0.4625</td>
</tr>
<tr>
<td>phyB2</td>
<td>6066</td>
<td>0.518</td>
<td>0.01</td>
<td>0.9112</td>
<td>0.9895</td>
<td>1.88</td>
<td>0.1701</td>
<td>0.6776</td>
</tr>
<tr>
<td>phyB2</td>
<td>6144</td>
<td>0.933</td>
<td>0.09</td>
<td>0.7661</td>
<td>0.9193</td>
<td>1.25</td>
<td>0.2645</td>
<td>0.6942</td>
</tr>
<tr>
<td>phyB2</td>
<td>6147</td>
<td>0.045</td>
<td>1.99</td>
<td>0.1579</td>
<td>0.4653</td>
<td>0.27</td>
<td>0.6005</td>
<td>0.8926</td>
</tr>
<tr>
<td>LD16</td>
<td>133</td>
<td>0.031</td>
<td>2.66</td>
<td>0.1030</td>
<td>0.4653</td>
<td>0.08</td>
<td>0.7738</td>
<td>0.9848</td>
</tr>
<tr>
<td>LD16</td>
<td>217</td>
<td>0.977</td>
<td>0.10</td>
<td>0.7492</td>
<td>0.9193</td>
<td>1.25</td>
<td>0.2635</td>
<td>0.6942</td>
</tr>
<tr>
<td>LD16</td>
<td>463</td>
<td>0.716</td>
<td>0.03</td>
<td>0.8709</td>
<td>0.9895</td>
<td>0.49</td>
<td>0.4836</td>
<td>0.8243</td>
</tr>
<tr>
<td>LD16</td>
<td>554</td>
<td>0.196</td>
<td>0.24</td>
<td>0.6229</td>
<td>0.8604</td>
<td>0.25</td>
<td>0.6163</td>
<td>0.8926</td>
</tr>
<tr>
<td>LD17</td>
<td>267</td>
<td>0.668</td>
<td>3.78</td>
<td>0.0518</td>
<td>0.3834</td>
<td>1.82</td>
<td>0.1775</td>
<td>0.6776</td>
</tr>
<tr>
<td>LD18</td>
<td>66</td>
<td>0.868</td>
<td>0.43</td>
<td>0.5131</td>
<td>0.8076</td>
<td>0.00</td>
<td>0.9877</td>
<td>0.9921</td>
</tr>
</tbody>
</table>

Continued on next page
<table>
<thead>
<tr>
<th>Fragment</th>
<th>Site</th>
<th>Frequency</th>
<th>$2\Delta_L$</th>
<th>p-value</th>
<th>q-value</th>
<th>$2\Delta_L$</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD18</td>
<td>204</td>
<td>0.175</td>
<td>0.01</td>
<td>0.9188</td>
<td>0.9895</td>
<td>0.11</td>
<td>0.7377</td>
<td>0.9773</td>
</tr>
<tr>
<td>LD20</td>
<td>441</td>
<td>0.129</td>
<td>0.97</td>
<td>0.3238</td>
<td>0.7157</td>
<td>4.12</td>
<td>0.0424</td>
<td>0.4625</td>
</tr>
<tr>
<td>LD20</td>
<td>471</td>
<td>0.830</td>
<td>0.44</td>
<td>0.5063</td>
<td>0.8076</td>
<td>0.00</td>
<td>0.9921</td>
<td>0.9921</td>
</tr>
<tr>
<td>LD23</td>
<td>220</td>
<td>0.371</td>
<td>0.01</td>
<td>0.9026</td>
<td>0.9895</td>
<td>3.36</td>
<td>0.0670</td>
<td>0.4688</td>
</tr>
<tr>
<td>LD23</td>
<td>311</td>
<td>0.375</td>
<td>0.00</td>
<td>0.9958</td>
<td>0.9958</td>
<td>2.57</td>
<td>0.1090</td>
<td>0.5723</td>
</tr>
<tr>
<td>LD23</td>
<td>520</td>
<td>0.004</td>
<td>1.82</td>
<td>0.1770</td>
<td>0.4653</td>
<td>0.04</td>
<td>0.8336</td>
<td>0.9885</td>
</tr>
</tbody>
</table>