

ORIGINAL ARTICLE

An excess of nonsynonymous polymorphism and extensive haplotype structure at the *PtABI1B* locus in European aspen (*Populus tremula*): a case of balancing selection in an obligately outcrossing plant?

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Here, we describe an unusually pronounced haplotype structure at the *PtABI1B* locus in the obligately outcrossing tree *Populus tremula*. Both nucleotide diversity and divergence at *PtABI1B* was low compared to other *P. tremula* genes suggesting that the gene is located in a region with a low mutation rate. Despite this, *PtABI1B* shows a very marked excess of nonsynonymous polymorphisms across the entire coding region and linkage disequilibrium (LD) extending across the entire *PtABI1B* region of approximately 2.6 kb. Such extensive LD is normally not seen in *P. tremula*.

The extensive LD at *PtABI1B* is caused by the presence of two distinct haplotypes. The haplotype structure is not caused by a lack of recombination in the region, because evidence of recombination can be detected. In addition, several statistical tests strongly reject neutrality for the *PtABI1B* region, suggesting that the unusual haplotype structure could be actively maintained by balancing selection.

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Introduction

Winter dormancy (endodormancy) is an important adaptive strategy that enables plants to persist during periods of stressful winter conditions, including freezing temperatures, dessication and ice encasement. The ability to develop dormancy determines whether perennial plants will survive winter and early spring without damage to shoot and flower buds and the initiation of dormancy therefore represents a critical ecological and evolutionary tradeoff between survival and growth in most perennial plants.

Endodormancy is preceded by a stage of ecodormancy, during which growth arrest is under the control of external environmental factors but growth can resume if conditions become favorable again. In most trees, ecodormancy is known to be induced by a shortening of the daily photoperiod mediated by the phytochrome system (Howe *et al.*, 2003). Recent evidence indicates that phytochrome involvement in photoperiod perception is mediated by plant hormones such as abscisic acid (ABA) or gibberellins (Eriksson and Moritz, 2002; Welling *et al.*, 2002; Bertrand and Castonguay, 2003). Increased ABA

levels have been shown to both induce and maintain bud dormancy in woody perennial plants (Wareing, 1956; Shimizu-Sato and Mori, 2001; Li *et al.*, 2003) and studies in both silver birch (*Betula pendula*, Li *et al.*, 2003) and black cottonwood (*Populus trichocarpa*, Rohde *et al.*, 2002) show that levels of ABA increase at the initiation of bud dormancy, thus suggesting a causal link between photoperiod perception, ABA regulation and the development of dormancy in perennial plants. Recent studies suggest, however, that changes in ABA sensitivity rather than variation in actual ABA levels may drive changes in dormancy status (Chen *et al.*, 2002).

Currently, little is known about variation in ABA responsiveness in natural plant populations or about naturally occurring variation in genes involved in regulating ABA signaling. In this paper, we analyze DNA sequence variation from a candidate gene (*PtABI1B*) involved in mediating ABA signaling, using samples from several populations of European aspen (*Populus tremula*). *PtABI1B* encodes a protein phosphatase 2Cs (PP2Cs), which have been shown to act as a negative regulator of ABA stress signaling pathways (Leung *et al.*, 1997; Leung and Giraudat, 1998). Quantitative trait locus (QTL) mapping studies have indicated that one of the poplar ABI homologs, *PtABI1B*, maps close to QTLs for both bud flush and bud set in *Populus trichocarpa* (Frewen *et al.*, 2000; Chen *et al.*, 2002). In addition, transcripts of *PtABI1B* have also been detected in buds at the time of bud set in the fall and transcription of *PtABI1B* also increase at the time of bud flush in the

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spring, suggesting that this gene could be actively involved in regulating bud phenology in *Populus* (Chen *et al.*, 2002).

We were initially interested in determining whether there was any evidence for adaptive genetic differentiation at *PtABI1B* across a latitudinal gradient representing growth seasons ranging from roughly 2–5 months. We have previously detected such adaptive differentiation at *phytochromeB2*, another candidate gene for dormancy initiation in *Populus* (Ingvarsson *et al.*, 2006). We failed to detect a pattern consistent with adaptive population differentiation at *PtABI1B*, but instead uncovered a very pronounced haplotype structure. Here, we further study this unusual haplotype structure by characterizing patterns of nucleotide diversity and linkage disequilibrium (LD) across the *PtABI1B* locus. We discuss some of the evolutionary forces that may be acting to maintain this haplotype structure in populations of *Populus tremula*.

Materials and methods

PtABI1B isolation

Primers to amplify *Populus* homologs of the *ABI1* genes from *Arabidopsis thaliana* were taken from Frewen *et al.* (2000). These primers were used to amplify a partial fragment of *PtABI1B* (Frewen *et al.*, 2000) from a cDNA library constructed from leaf tissue. The resulting PCR product was cloned into the pCR2.1 vector using a TA-cloning kit from Invitrogen (Carlsbad, CA, USA) and sequenced using BigDye chemistry (Applied Biosystems Inc., Foster City, CA, USA) on an ABI377 automated sequencer at the Umeå Plant Science Centre sequencing facility. The 5' portion of the transcript was obtained through 5' RACE using a GeneRacer Kit from Invitrogen and new gene-specific primers were developed from the sequenced transcript. Primers were designed to amplify the complete *PtABI1B* gene. The homologous sequence from *P. trichocarpa* was obtained by BLAST searches of the assembled genome sequence of *P. trichocarpa* available at <http://genome.jgi-psf.org/>. Although the *Populus* homologs of the *ABI1* genes are made up of a small gene family (unpublished), a BLAST search of the *P. trichocarpa* genome sequence suggest that the *PtABI1B* gene is a single-copy gene.

Sequence analysis

The *PtABI1B* gene was amplified from a total of 22 individuals sampled from four localities in Europe. Samples were taken within a few kilometers of Besançon in eastern France (FRA, four individuals), Klagenfurt in Southern Austria (AUT, six individuals), Färjestaden in Southeastern Sweden (SWE-S, six individuals) and Umeå in northern Sweden (SWE-N, six individuals, see Ingvarsson (2005) for more details about these populations). Because *P. tremula* is diploid and outcrossing, these 22 individuals represent 44 independent haplotypes. PCR products were cloned into the pCR2.1 vector (Invitrogen) and at least five, and often more, clones of each fragment were sequenced to obtain both haplotypes from an individual. To ensure that we were not inadvertently studying a pair of recently duplicated paralogs, we studied a few individuals in greater detail. In these individuals, a large number of clones (>20)

were sequenced and we also performed restriction digests to check for restriction patterns inconsistent with a single, diploid locus. In no case did we find more than two haplotypes among the sequenced clones and our restriction analyses did not find any evidence suggesting a recent duplication of *PtABI1B*. We therefore conclude that *PtABI1B* is a single-copy gene also in *P. tremula*.

Sequences were verified manually and contigs were assembled using the 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 (AM690392–AM690435).

Estimates of nucleotide polymorphism, LD and statistical tests of neutrality were obtained using a computer program written in C++ based on the publicly available C++ class library libsequence (Thornton, 2003). Significance of statistical tests of neutrality were evaluated by determining the null distribution for each test statistic under neutrality, by simulating 10^5 neutral genealogies using Richard Hudson's ms program (available at <http://home.uchicago.edu/~rhudson1/source/mksamples.html>). Briefly, simulations were conditioned on the original sample configuration, sequence lengths and the number of segregating sites observed. Population structure is known to influence both power and rejection rates of statistical tests of neutrality (Przeworski, 2002) and simulations were therefore run with samples taken from a subdivided population. The migration parameter, $M = 4Nm$, was set so that the expected value of $F_{ST} = 1 - \pi_{within} / \pi_{total}$ (Charlesworth, 1998) matched that observed in the sample. Population structure in the simulations was assumed to follow Wright's Island Model with a total number of populations equal to 20. Finally, we also included recombination in our coalescent simulations. We did this by calculating the per-base pair recombination rate (ρ) estimated using the method of Hudson (1987). Statistical tests of neutrality generally gain power as the recombination rate increases (Wall, 1999). We therefore ran our coalescent simulations using two recombination rates, ρ or $\rho/2$. They gave the same results and we therefore only report the results from the simulations using the more conservative recombination rate ($\rho/2$).

We also used a sliding-window approach to study how patterns of polymorphism varied across the *PtABI1B* gene region. To determine the significance of summary statistics calculated in individual window segments, we used coalescent simulation of an infinite island model with the same number of alleles that were present in the four populations sampled (8, 12, 12 and 12, for a total of 48). Simulations were run conditional on the number of segregating sites observed in each window segment and the migration parameter, $M = 4Nm$, was set so that the expected F_{ST} matched the estimate of F_{ST} across the entire *PtABI1B* gene.

LD extends only a few hundred base pairs in *P. tremula* (Ingvarsson, 2005), and signals of balancing selection are expected to affect only small genomic regions (Charlesworth, 2006). We therefore used a fixed window size of 200 bp in our sliding window analyses; smaller window sizes resulted in zero polymorphism in many windows and with larger windows one runs the risk of averaging over too many sites with independent evolutionary

histories, thereby reducing the power to detect deviations from neutral expectations. The sliding window analysis is clearly exploratory and should be viewed as method for identifying regions worth studying in greater detail. We therefore do not correct for multiple testing in the analysis.

Results

Nucleotide polymorphism and divergence

We obtained the complete coding sequence and all intervening introns of *PtABI1B* from 44 haplotypes. The total aligned region, including indels, covered 2669 bp. We found a total of 32 segregating sites and overall, sequence diversity was low, $\pi = 3.2 \times 10^{-3}$ and $\theta = 3.0 \times 10^{-3}$, compared to other genes in *P. tremula*, (Ingvarsson, 2005; Ingvarsson *et al.*, 2006; Table 1). Synonymous site diversity was also two- to fivefold lower compared to previous estimates from *P. tremula* (range of π_s 11.1–30.3 $\times 10^{-3}$, Ingvarsson, 2005). Interestingly, polymorphism at noncoding sites, in introns and the 5'-untranslated region, is approximately threefold greater than diversity at silent sites ($\pi_{nc} = 3.59 \times 10^{-3}$). Jukes–Cantor corrected divergence from the outgroup species *Populus trichocarpa* is 0.0152, and this is also two to five times lower than other *P. tremula* genes, where divergence from *P. trichocarpa* averages 0.0435 (Ingvarsson, 2005). The low levels of silent polymorphism and divergence therefore suggest that *PtABI1B* could be located in a genomic region with an unusually low mutation rate.

The most striking feature of the polymorphism data at *PtABI1B* was the marked excess of polymorphism at nonsynonymous sites. In total, 18 of 20 polymorphic sites in the coding region are nonsynonymous (Table 2). Also, nucleotide diversity at nonsynonymous sites was $\pi_a = 3.5 \times 10^{-3}$ compared to $\pi_s = 1.10 \times 10^{-3}$ at synonymous sites, yielding a π_a/π_s ratio of 3.18 (Table 1). A McDonald–Kreitman (MD) test, which contrasts the rates of divergence with the levels of standing polymorphism within a species at synonymous and nonsynonymous sites, was performed using *P. trichocarpa* as an outgroup (McDonald and Kreitman, 1991). The MK test shows a significant deviation from neutral expectations at *PtABI1B* ($P = 0.036$). Such a deviation from neutral expectations could be caused either by an excess of nonsynonymous mutations or by a deficit of synonymous mutations. If polymorphism and divergence at synonymous sites is contrasted with polymorphism and divergence at noncoding sites in the *PtABI1B* region,

there is no evidence for any departure from neutrality ($P = 0.441$). On the other hand, a similar test contrasting nonsynonymous and noncoding sites approaches significance ($P = 0.052$), and a contrast between nonsynonymous and all silent sites in the *PtABI1B* region (synonymous + noncoding) was significant ($P = 0.042$), suggesting that the significant MK test at *PtABI1B* is caused by an excess of nonsynonymous polymorphisms segregating within *P. tremula* rather than a deficit of polymorphisms at synonymous sites.

Haplotype structure and LD

Two nonsynonymous mutations in exon 3, separated by 15 bp, are linked in repulsion (Figure 1). These two sites (E321K, nucleotide site 1221 and N325K, nucleotide site 1235) are also in LD with several other synonymous and nonsynonymous variants across the entire coding sequence and define two distinct clades at *PtABI1B* (Figures 1 and 2). There are also five nonsynonymous mutations (including the E321K and N325K mutations), a 170 bp deletion in intron 4 (IND1) and additional four mutations in intron 4 that are fixed between the two clades. All but one of the 44 sequenced alleles carry either of these two variants. In addition, one sequence appears to be a recombinant between the two major clades (Figure 1). Although the two clades contain roughly similar levels of sequence diversity ($\pi_{Hap1} = 0.0014$ and $\pi_{Hap2} = 0.0011$), patterns of polymorphism differ substantially; clade 1 has an excess of nonsynonymous polymorphisms ($\pi_a/\pi_s = 2.43$), whereas clade 2 has an excess of synonymous polymorphisms ($\pi_a/\pi_s = 0.67$).

A sliding window analysis shows two peaks of high polymorphism, one located in exon 2 and one in exon 3 (Figure 3). The peak in exon 3 occurs in the vicinity of the two nonsynonymous variants that define the two major clades at *PtABI1B* (Figures 1 and 2). Intraclade polymorphism in these regions is relatively low and the excess diversity seen in the total sample thus represents variants that are fixed between the two clades (Figure 3). These regions are also characterized by a frequency

Table 2 Polymorphism and divergence at nonsynonymous, synonymous and noncoding sites

	Replacement	Silent	Noncoding
Fixed	9	6	16
Polymorphic	18	2	12

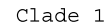
Table 1 Estimates of nucleotide variation and statistical test of neutrality at *PtABI1B*

Grouping	Alleles	Number of sites			Polymorphism ($\times 10^{-3}$) (\pm s.e.)							Statistical tests of neutrality		
		Total	Silent	Replacement	S	θ_W	π_{tot}	π_{sil}	π_{repl}	K_a/K_s	Divergence	D_{Tajima}	$F_{Fu\&Li}$	Z_{ns}
Clade 1	30	2651	354.5	1265.5	21	0.0020	0.0016	0.0007	0.0017	0.556		−1.089*	−1.191	0.178***
Clade 2	13	2488	355.0	1265.0	7	0.0009	0.0011	0.0014	0.0009	0.655	0.0041 ^a	0.648	0.355	0.353***
All	44	2669	354.6	1265.4	32	0.0030	0.0032	0.0011	0.0035	0.586	0.0139 ^b	0.239	−0.027	0.196***

^aNet divergence between clades 1 and 2.

^bNet divergence between *Populus tremula* and *Populus trichocarpa*.

* $P < 0.05$, *** $P < 0.001$.



Both nucleotide diversity and divergence from *P. trichocarpa* were three to five times lower at *PtABI1B* (Table 1) than at other genes in *P. tremula* (Ingvarsson,

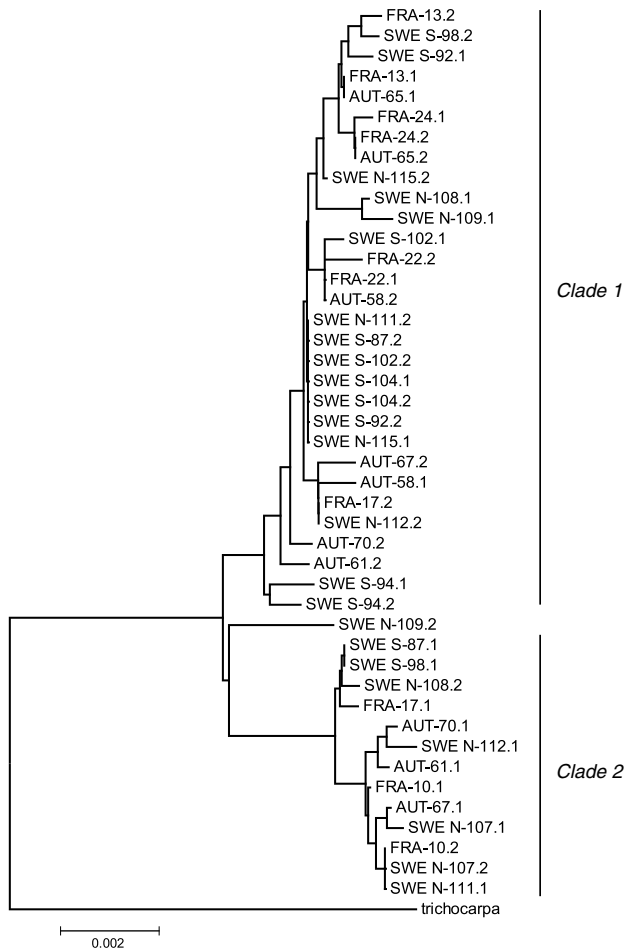


Figure 2 Gene genealogies for 44 alleles of *PtABI1B* in *Populus tremula*. *PtABI1B*-C1 and *PtABI1B*-C2 refer to the two major clades identified at *PtABI1B*. The genealogy was constructed using the neighbor-joining method with *p*-distances in MEGA 3.0.

2005), making it likely that *PtABI1B* is located in a region of the genome characterized by a low mutation rate. Despite the overall low levels of polymorphism at *PtABI1B*, there is a marked excess of nonsynonymous polymorphisms in the coding region of *PtABI1B* (Table 2). The polymorphism data are organized into two very distinct allelic clades (Figure 1), which extend across much of the gene region. These two clades are defined by two nonsynonymous variants located in the PP2C domain of *PtABI1B* that constitutes the most important active domain of the *PtABI1B* protein (Rodriguez, 1998). These variants are also in LD with several other nonsynonymous variants across the entire *PtABI1B* gene (Figure 1) and with a 170 bp deletion in intron 4 (IND1).

A sliding window analysis shows two peaks of elevated nucleotide polymorphism at *PtABI1B* (Figure 3). The regions of elevated polymorphism are also associated with frequency spectra that are skewed toward an excess of mutations occurring at intermediate frequencies (Tajima's $D > 0$, Figure 3). Population subdivision or admixture can result in spurious haplotype structuring and positive Tajima's D . However, the pattern observed at *PtABI1B* is not likely to have been caused by population subdivision. First, the two major clades at *PtABI1B* are present in roughly equal frequencies in

the four populations sampled (frequencies of clade 1 in the four populations are FRA = 0.70, AUT = 0.70, SWE-S = 0.83 and SWE-N = 0.58). This is also evident from the low genetic differentiation observed at *PtABI1B* ($F_{ST} = 0.004$), which is actually lower than other genes surveyed in the same set of populations, arguing against population subdivision as a cause of the haplotype structure (Ingvarsson, 2005; Ingvarsson *et al.*, 2006). Admixture could also result in strong haplotype structure and LD. However, effects of recent admixture should be evident throughout the genome, yet there are no such indications and population structure is overall very low in *P. tremula* (Ingvarsson, 2005; Ingvarsson *et al.*, 2006). It is possible that one of the haplotypes has introgressed through hybridization with a closely related species. *P. tremula* is known to hybridize with *P. alba* in regions where their distributions overlap (Lexer *et al.*, 2005). However, gene flow in hybrid zones between the two species appears to be biased toward introgression from *P. tremula* to *P. alba* (Lexer *et al.*, 2005). Whether one of the allelic clades arose through introgression from *P. alba* needs further investigation, preferably by sequencing of the *PtABI1B* gene also in *P. alba*. Nevertheless, although hybridization can help explain the origin of the two different clades, it still does not explain why the two haplotypes appear to be maintained in *P. tremula*.

LD does not decrease with distance at *PtABI1B* where it decreased to negligible levels in <500 bp in all other genes in *P. tremula* that have been studied to date (Ingvarsson, 2005; Ingvarsson *et al.*, 2006). These observations make it likely that some form of natural selection is the cause of the strong haplotype structure at *PtABI1B* in *P. tremula*. Selective sweeps can transiently increase levels of LD, although this should also lead to a reduction in levels of polymorphism in surrounding areas (Nielsen, 2005; Charlesworth, 2006). Even a partial sweep of one of the clades is thus inconsistent with the data, as synonymous site diversity is roughly equal in the two clades, making it unlikely that one of them have recently increased in frequency (Charlesworth, 2006). Balancing selection, on the other hand, can maintain polymorphisms for extended periods of time, resulting in the accumulation of mutations at sites in close proximity of the balanced polymorphism, yielding a frequency spectrum that is skewed toward alleles at intermediate frequencies (Charlesworth, 2006). Innan and Tajima (1999) showed that if balancing selection is maintaining two distinct alleles at a locus at roughly constant frequencies, the sum of pairwise differences within the two classes is roughly constant and equal to θ , regardless of the strength and pattern of selection. The sum of silent site diversity within the two clades at *PtABI1B* equals $\pi_{sum} = 0.0026$, which is not significantly different from the silent site diversity observed in the total sample ($\pi = 0.0029$). A model of constant balancing selection thus appears to provide a possible explanation for the *PtABI1B* data.

A closer examination of the high π_a/π_s ratio at *PtABI1B* show that it is partially explained by four nonsynonymous mutations that are fixed between the two major clades. In addition to these nonsynonymous mutations, several synonymous mutations and the deletion in intron 3 are also fixed between the two clades. The peculiar haplotype structure seen at *PtABI1B* thus has all the hallmarks of a balanced polymorphism, including

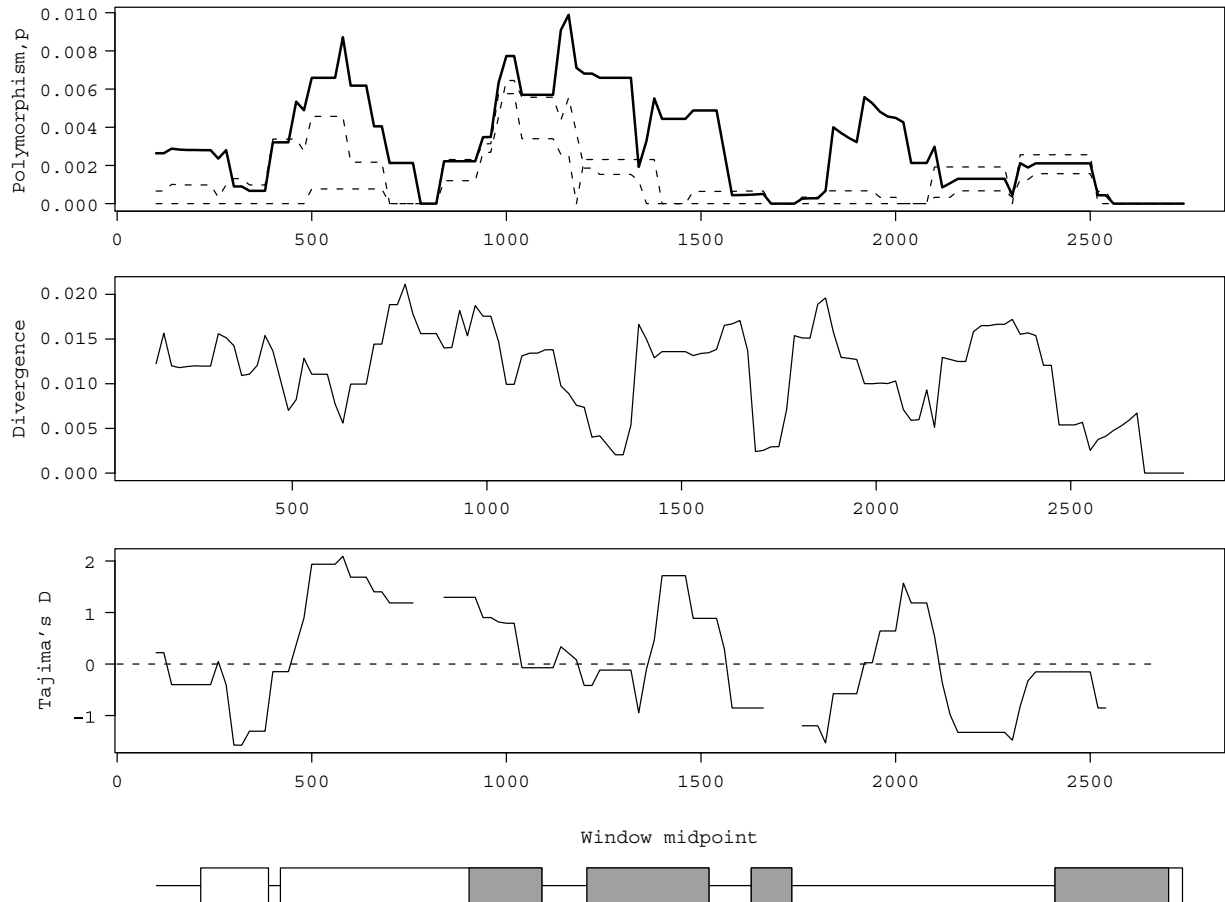


Figure 3 Sliding window plot of nucleotide polymorphism, π , divergence from *P. trichocarpa* (middle) and Tajima's D (bottom). A window of width 200 bp was moved along the sequence in 20 bp increments and statistics were calculated for each window segment. Exon-intron structure of *PtABI1B* is indicated by black boxes (exons) and thin lines (introns) at the bottom of the graph.

distinct allelic clades, high LD and an excess of sites at intermediate frequencies.

The extent to which a genomic region surrounding a balanced polymorphism is affected critically depends on the local recombination rate. Nordborg and Innan (2003) showed that the likelihood of observing the signature of balancing selection is a function of both the population mutation rate, $\theta = 4N\mu$, and population recombination rate, $\rho = 4Nr$. The mutation rate determines the amount of neutral diversity in a population, whereas the recombination rate determines the degree of associations among sites. In highly selfing species, the recombination to mutation ratio will be low because the effective recombination rate is low in highly inbred genomic backgrounds (Nordborg, 2000). The signature of balancing selection is therefore expected to extend over large distances in inbred species (Nordborg *et al.*, 1996; Charlesworth, 2006). In an outcrossing species, such as *P. tremula*, the recombination to polymorphism ratio is expected to be high and all but the most tightly linked sites will be uncoupled from a balanced polymorphism and will effectively behave as neutral sites. The power to detect balancing selection is therefore low in most outcrossing species (Nordborg *et al.*, 1996; Charlesworth, 2006). It is thus not surprising that, besides the notable exceptions of self-incompatibility loci (Charlesworth *et al.*, 2003), relatively few cases of balancing selection

have been documented in outcrossing plants (Charlesworth, 2006). Recombination does not appear to be unusually low in the *PtABI1B* region; sequence-based estimates of the recombination rate at *PtABI1B* (using the method of Hudson (1987)) yields an estimate of the per site recombination rate of $\rho = 2.39 \times 10^{-3}$ /bp, which is in line with estimates from other genes in *P. tremula* (Ingvarsson, 2005). Therefore, even if the putative signal of balancing selection can be detected across most of the 2.6 kb region of the *PtABI1B* gene, the likelihood of detecting this unusual haplotype structure without actually targeting the core PP2C region of *PtABI1B* appears to be low. This is also evidenced by the fact that while there is an excess of sites at intermediate frequencies in the core PP2C regions, when averaged across entire gene there is no such excess (Table 1). This likely occurs because the evolutionary histories of sites in the extremes of the *PtABI1B* gene are decoupled from that of the sites in the core PP2C region.

Although several signs suggest that the *PtABI1B* haplotype structure is being maintained by selection, we can only speculate the functional basis for the polymorphism. Data from homologous genes in *Arabidopsis thaliana* suggest that sequence variants in these genes can result in either increased or reduced responsiveness to ABA (Merlot *et al.*, 2001). In *A. thaliana* both mutants from which the *ABI1* and *ABI2* genes were

initially described, carry the same Gly to Asp substitution in the PP2C domain of the *ABI* protein (Leung *et al.*, 1997). These variants are both partially dominant and result in reduced ABA sensitivity of the mutant plants (Leung *et al.*, 1997). The homologous amino acid is conserved in our sequences from *P. tremula*, but it is interesting to note that the two amino-acid polymorphisms that make up the two distinct haplotypes at *PtABI1B* are located only 100 bp downstream from the site of the *A. thaliana* mutations. The physical location of the two variants (E321K and N325K), in the core PP2C region, suggest that they could be involved in altering sensitivity to ABA. However, more work is needed to work out the functional significance of these variants and to understand possible targets of balancing selection.

Charlesworth (2006) argued that short-term balancing selection is probably far more likely than the more well-studied cases of long-term balancing selection, such as those associated with self-incompatibility loci or certain disease resistance polymorphisms. The results presented here highlight the difficulties inherent in locating possible candidates for 'weak' balancing selection in outcrossing species. Unless selection is strong and/or the region is associated with a reduced recombination rate, the genomic signal of balancing selection will be weak and such regions will be hard to identify through various test of selection (Charlesworth, 2006). Although weak balancing selection may be hard to detect at the genome level, such sites could have a significant impact on quantitative variation seen in natural populations, including variation in many fitness-related traits. How much of the standing genetic variation in quantitative traits is due to weak balancing selection has remained controversial (Johnson and Barton, 2005). However, with the accumulation of genomic data from a large number of species, we are now in the position where it is possible to start addressing questions on the genetic basis of quantitative variability in natural populations.

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