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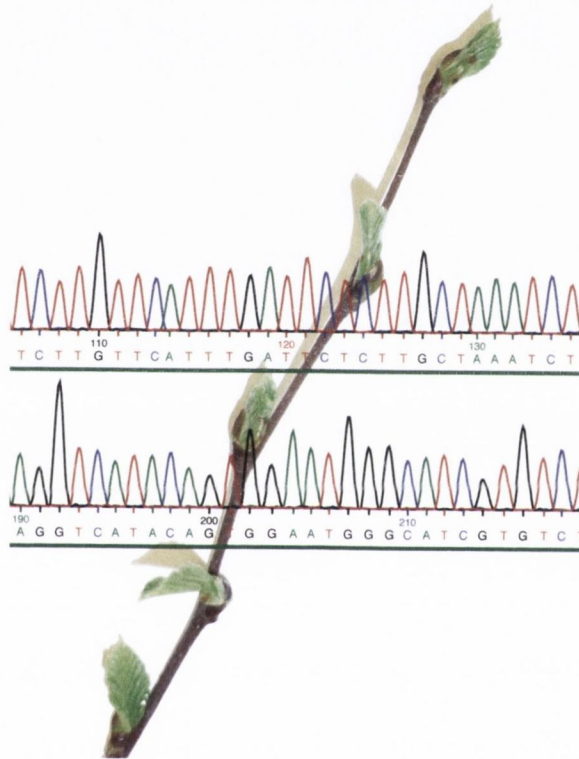
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Insights into the phenotypical and genetic mechanisms regulating bud burst of temperate trees in relation to climate change



A dissertation submitted to the University of Dublin for the degree of
Doctor of Philosophy

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April 2012



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SUMMARY

The work presented here provides a combination of different experimental techniques and different analysis methods in order to gain a deeper understanding of the mechanisms involved in bud burst of temperate trees. Two tree species native to Europe were the subjects of investigation: European aspen (*Populus tremula* L.) and downy birch (*Betula pubescens* Ehrh.). The mechanisms behind the trait of bud burst in trees were considered at two levels: the phenotypic and genotypic mechanisms.

In the first chapter, an extensive literature review is provided explaining the topics that are the focus in the three following chapters, including a state of the art discussion of current research for each topic. The final two sections of the introductory chapter provide a detailed description of the two study species, and an outline of the main aims and objectives of the work contained in the subsequent results chapters.

In brief, Chapter 2 reports the investigations on the timing of bud burst using an experimental approach. The aim was to quantify the effects of chilling duration and photoperiod on the percentage and timing of bud burst in *P. tremula* and *B. pubescens*, using clones and controlled environment experiments. The experiments were carried out over two separate seasons with varying conditions. The results showed that chilling duration had a significant effect on the timing of bud burst for both species, however the nature of the response differed between the species in speed and percentage of bud burst. For both species the photoperiod effect was weaker for longer chilling durations, which in natural conditions would imply a loss of a 'security factor' against late frost damage for newly emerged leaves. The results showed potential for the use in a modelling analysis.

In Chapter 3, a modelling approach was used to find the parameters that determined bud burst in the experiments described in Chapter 2. Besides a fitted thermal time model for the data obtained, the further investigations in the data resulted in an interesting insight of how the effects of experimental conditions on the timing of bud burst were brought about. Using

the novel approach of comparing the distributions of bud burst per tree, it was discovered that not only the variation between the experimental conditions played a role, but also the tree-to-tree variation and even the bud-to-bud variation within a single tree. These insights provide useful information for modellers, and thus for predicting future changes in bud burst with climate change.

Finally, in Chapter 4, the reader is presented with bud burst on an entirely different scale and enters the molecular world. In Chapter 1 and 2, tree clones were used in order to exclude genotypic variation in the response, while in this chapter the aim was exactly to find that genetic variation, among natural populations of *P. tremula*. A set of candidate genes was identified, putatively involved in dormancy release and bud burst. The genetic variation between the European aspen populations, with samples actually originating from an extensive range across Europe, was determined and some indications were detected for relationships between genetic variation in phenology-related genes and natural gradients (clinal variation).

Except for Chapter 1, each of the chapters start with a short introduction to the study presented and the specific aims. Subsequently, a brief explanation is given of the methods used: the experimental design and statistical methods in Chapter 2 and Chapter 3, and the laboratory techniques and analysis methods in Chapter 4. In each of the chapters, the findings are described and demonstrated in the results section, after which a discussion section clarifies the results by comparing with previous studies, discussing the implications and suggesting directions for future research.

A final discussion chapter (Chapter 5) can be found at the end of the thesis in order to draw together the genetic and phenotypic findings of this study.

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Annelies

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Chapter 1:

Tree phenology under climate warming conditions in Ireland and Europe: genotypic and phenotypic aspects

1.1 Introduction

1.1.1 Trees

Trees and forests are extremely important for human life on earth. Since they evolved (an event which happened several times in the scope of evolution, e.g. Arber 1928), trees have been involved in shaping the ecology and biodiversity of our planet, for instance by providing large amounts of O₂ for other developing life forms on earth. They have played an important role in the development of human civilisation in providing timber, paper, food, energy etc. Even today new uses are being discovered, such as Taxol, which is used in the treatment of certain types of cancer and developed from a plant alkaloid found in yew trees (*Taxus brevifolia* Peattie, Wani et al. 1971). Forests play a vital role in mitigating CO₂ emissions by sequestering carbon in the biomass of trees, and have environmental benefits such as protection against flooding, bioremediation, nutrient cycling, and providing a habitats for wildlife, including endangered species (Bradshaw et al. 2000, Taylor 2002). For use as research objects, several differences between trees and herbaceous plants need to be taken into account, mainly: (1) their longevity, (2) their supporting and hydraulic structure (wood), and (3) their mechanisms to cope with variable biotic and abiotic stresses during their lifespan (e.g. phenological responses). Understanding the particular systems and mechanisms of tree growth and survival will help determine ways in which trees can be used more efficiently for various environmental and economic purposes (Brunner et al. 2004, Aitken et al. 2008), which is particularly important for the cultivation of trees in the light of climate change. Particularly integrated with other farm activities (agroforestry), tree cultivation has the potential to contribute to limiting deforestation, in situ conservation, and to an increase in the use of renewable energy sources (Nair et al. 2009, World Bank 2009).

1.1.2 *Climate change*

Climate plays a major role in the survival and distribution of species and populations. From paleoclimatic data (inferred from proxy sources e.g. dendrochronological measurements, fossils, ice cores, corals, or ocean sediment composition), it is clear that over earth's geological history the planet has experienced large swings in climate, which resulted in periods of extinction but also in the diversification of life, and on a smaller scale, distributional changes also occurred (IPCC 2007, Juckes et al. 2007). After the Last Glacial Maximum (LGM) for instance, postglacial range expansions of many European tree species occurred by migration from southern regions and a number of marginal refugia (Pearson 2006).

According to the Intergovernmental Panel of Climate Change (2007), the definition of climate change is 'a change in the state of the climate that can be identified by changes in the mean and/or the variability of its properties, and that persists for an extended period, typically decades or longer, [...] whether due to natural variability or as a result of human activity'. The world's recent climate change resulting from an increase in greenhouse gas concentration, and in particular from CO₂, has been causing an increase in average global temperature, and a more frequent occurrence of extreme events (IPCC 2007). Species distributions and consequently the composition of ecosystems are affected by climate through impacts on the phenology, physiology and interactions of plants (Walther 2003). With a rapidly changing climate and the introduction of trees to alien pests and diseases, the survival of tree populations may depend on their ability to adapt or to migrate (Aitken et al. 2008). There is growing evidence that recent climate change is causing detectable changes in ecosystems, such as species distribution shifts and changes to forest species composition (Walther et al. 2002). Although temperature records can now easily be determined and compared with plant distribution maps, the actual relationship between climatic limits and plant distribution is not quite as straightforward as initially assumed. As pioneering eco-physiologist Eilif Dahl stated in 1998, "the real question we have to ask is: in which way does temperature affect plant performance?".

1.1.3 Phenology

In temperate and boreal regions, trees are able to survive harsh winters and be prepared for more favourable growing conditions in spring through phenological (regularly recurring) mechanisms such as dormancy (temporary growth reduction) and cold hardiness (increased resistance to cold; Crabbé 1994). These traits are adaptive, which allows species and populations to migrate and colonise new areas. In this way, it is possible for the northern limits of boreal forests and altitudinal treelines to shift northwards or to higher altitudes when climatic changes occur, which happened after the LGM, between 26 500 and 20 000 years ago (Crawford and Jeffree 2004, Pearson 2006). Phenological events in the lifespan of temperate trees include flowering, bud burst and leaf senescence, which are controlled by a complex interaction of several environmental cues, mainly temperature (degree of winter chilling as well as spring temperature) and photoperiod (day length relative to night length; Chuine and Cour 1999, Crabbé 1994).

1.1.4 Genotypic and phenotypic research

Individuals can respond to changes in their environment through phenotypic plasticity, while populations and species can respond through genetic adaptation over several generations. Because of the strong link between temperature and phenology, and because phenology (mainly through flowering time) has an effect on reproduction, climate change and rising temperatures in particular, has the ability to impose natural selection on phenological traits (Franks 2007). Plant scientists now have a great challenge in understanding how these genotypic and phenotypic responses interact, in order to predict the impacts of climate change on crops, forestry and natural ecosystems, and respond accordingly. It is necessary to study these responses at all scales, from molecular and cellular up to organ-, plant- and biome level; and in woody as well as herbaceous plants (Morison and Morecroft 2006). Genetic research in plants historically focused on the annual, herbaceous model plant *Arabidopsis thaliana* (L.) Heynh. but in order to understand the unique processes in trees such as dormancy and secondary wood formation, it was necessary to identify a woody model

organism. The model species proposed was *Populus trichocarpa* Torr. and A.Gray, and the entire *Populus* genus has been particularly useful for genetic methods (Bradshaw et al. 2000, Taylor 2002).

1.2 Changes in tree phenology as a result of climate warming

1.2.1 What is Phenology?

The term 'phenology' is derived from the ancient Greek word 'phainesthai' (to show or to appear) and was first used in a scientific context by Belgian botanist Charles Morren in 1849 (Demarée and Rutishauser 2009). A working group of the International Biological Programme defined phenology as "the study of the timing of recurrent biological events, the causes of their timing with regard to biotic and abiotic forces, and the interrelation among phases of the same or different species" (Lieth 1974). Phenological research focuses mainly on the timing of changes in recurring developmental or behavioural phases of organisms. In plant phenology, events such as the start of growing season or the initiation of developmental phases such as bud set, bud burst, or flowering are examined and usually related to climatic variables (Roy and Sparks 2000, Donnelly et al. 2009). Besides ground observations, continuous changes on a larger scale, such as vegetation growth can be observed by remote sensing (Davison et al. 2011), and even measurement of the NEP (Net Ecosystem Productivity) can be used to determine seasonality (Richardson et al. 2010). Remote sensing of vegetation phenology can be carried out by measuring the 'greening' of a canopy or landscape through satellite images, web cams or by measuring NDVI (Normalised Difference Vegetation Index) to estimate FAPAR (Fraction of Absorbed Photosynthetically Active Radiation; O'Connor et al. 2010). In plants, phenological changes due to climate change become apparent through changes in the length of growing season (LGS). Therefore, LGS can be used as an indicator of climate change. The main factor that plays a role in this relationship is an earlier onset of spring through a rise in temperature, while the timing of autumn events is less consistent in showing changes (Menzel et al. 2000, Chmielewski and Rötzer 2001, Donnelly et al. 2006).

Phenological studies have a long history. For instance, the Japanese have an ancient custom of celebrating the flowering of the cherry trees to welcome spring and records of these dates have been kept since the 9th century (Arakawa 1956). In Europe, Carolus Linnaeus (Sweden, 1707-1778) and Robert Marsham (UK, 1708-1797) were the first to systematically record dates of flowering times and other indications of spring (such as bird arrival dates and dates of first butterflies), and are thus considered the 'fathers' of phenology (Sparks and Lines 2008). But mainly in recent years, phenology has been gaining a newfound interest because of climate change. For instance, the most recent IPCC Fourth Assessment Report (IPCC 2007) featured a pan-European study that reported advanced spring across Europe between 1971 and 2000 (Menzel et al. 2006) as an indicator of climate change. The numerous monitoring websites and publications highlight the importance of this science (1) in convincing policy makers that climate change is having a measurable impact on the environment and (2) for estimating the effects on the survival, behaviour, distribution and interactions of species - the 'fingerprints' of climate change (Parmesan and Yohe 2003, Walther 2003). Changes in phenology and the length of growing season are very likely to have considerable consequences for agriculture, forestry, biodiversity and human health.

Recent climate change is caused by increased emission of greenhouse gases, in particular CO₂, due to human activities (IPCC 2007). The consequences are rapidly raising temperatures, changes in precipitation patterns, and an increase in the frequency of extreme weather events in many regions. The current and predicted results include sea level rise and major impacts on ecosystems, through changes in physiological responses in plants and animals (IPCC 2007). Changes of phenological events are examples of how these physiological changes can become manifested, and not only in plants but also for insects and birds there is a strong association between development and temperature (Donnelly et al. 2011). For instance, the earlier first appearance of butterflies in the UK (Roy and Sparks 2000) and bees in Spain (Gordo and Sanz 2006) have been linked to temperature rises, and the egg laying dates of a great tit population in the UK have been brought forward by two weeks over a 47-year period correlated with climate change (Charmantier et al. 2008). These phenological changes across different trophic levels do not occur simultaneously and can

result in spatial (e.g. range shifts) or temporal mismatches (timing of phenological events). For instance, forest caterpillars have an optimal hatching time to ensure maximal leaf availability for the population, and any asynchrony in this insect-plant interaction can cause major decrease in fitness for the caterpillar population (van Asch and Visser 2007).

1.2.2 Plant phenology in Europe

Within Europe, a wide range of climatic conditions, types of land use, levels of exposure, and inclinations can be found, and therefore plant phenology can vary significantly according to all those factors. The geographical distribution of the timings of phenological events forms the basis for investigating the relationships between phenology and climate variability. Rötzer and Chmielewski (2001) computed the first phenological maps of Europe, and since then remote sensing, satellite imaging and modelling have been causing a development in mapping phenology (e.g. Figure 1.1, Jung et al. 2008, Jeanneret and Rutishauser 2010).

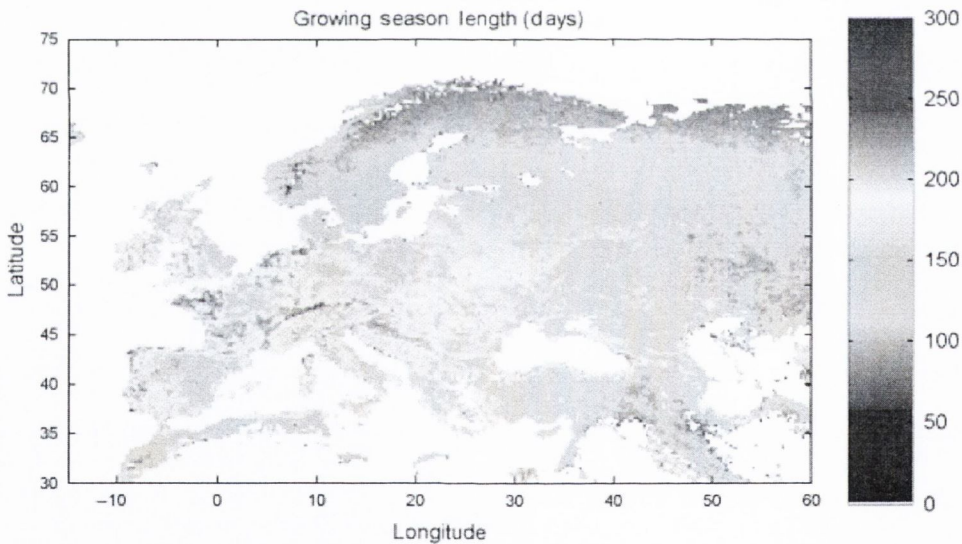


Figure 1.1 Map of the mean growing season length (1998-2002) based on cumulative FAPAR calculations of the growing season (from Jung et al. 2008).

In Europe, the growing season has lengthened by 10.8 days between the 1960s and 1990s, mainly due to an advancement of spring events (Menzel and Fabian 1999). But although the climate is warming on a large scale, change at a local level can be more variable and difficult to detect. Assessing changes in the phenology of plants can give us indications of climate change impacts, in particular increasing temperature, at a local level. Long-term datasets of

20 years or longer are generally recommended to detect phenological trends (Sparks and Menzel 2002), although Amano et al. (2010) have used hierarchical models and short-term records from multiple sites to estimate a 250-year index of first flowering. In 1959, a European network (International Phenological Gardens, IPG) was established for observations of deciduous trees, shrubs and conifers (Schnelle and Volkert 1974). In all gardens (78 gardens in 2008), genetically identical plants of up to 23 species are planted in order to compare the timing of phenological phases of the plants.

The most common phases that are monitored are the beginning of leaf unfolding, the beginning of flowering, first ripe fruits, autumn colouring, and leaf fall. The IPG network is the most extensive observation series in Europe. The IPG studies show that the growing season in 1993 was 10.8 days longer than in 1959, but the rate of change can vary between species, locations and time of year. In Western Europe, spring phenology is changing faster than in Scandinavia, for instance (Menzel and Fabian 1999, Menzel 2001, Chmielewski and Rötzer 2001). Besides the IPG network in Europe, many countries have their own smaller-scale national network (e.g. Natuurkalender in the Netherlands, Nature's Calendar in the UK, Nature Watch in Ireland) and also outside Europe networks are bringing together scientific and/or 'citizen-scientific' observations of phenophases throughout the year for a wide range of plant and animal species (e.g. USA-npn and Project BudBurst in the US, and PlantWatch in Canada).

1.2.3 Plant phenology in Ireland

Four IPGs were established in Ireland in the 1960's (National Botanic Gardens, Dublin; JFK Arboretum, Wexford; Johnstown Castle, Wexford and Valentia observatory, Kerry) and have been providing data since then. More gardens were added to the network in 2004 (Armagh Observatory), 2007 (Glenveagh National Park) and 2009 (Ballynahinch Castle, Markree Castle, Baronscourt, Mount Stewart, Ballyhaise College, Williamstown House, Carton Estate, Altamont Garden, Birr Castle and Millstreet Country Park). In addition, a Native Phenology Network was established in 2009, which is monitoring six native Irish tree species in 13

gardens (Proctor and Donnelly 2009). The four original phenological gardens are the only systematic long-term records for plant phenology in Ireland and have already been used successfully as indicators for spring warming (Donnelly et al. 2006). Here, the beginning of the growing season of some tree species has advanced across all four sites over the 30-year period of 1970-2000. The growing season has extended by 9 days for *Betula pubescens*, 3 days for *Fagus sylvatica* L. and 7 days for *Tilia cordata* Mill., for every rise of 1 °C in annual temperature. The signal is less clear in autumn, so the length of growing season has mainly increased due to earlier leafing (Sweeney et al. 2002, Donnelly et al. 2006).

1.2.4 Phenological models

Various models using climatic variables such as temperature and photoperiod have been developed for the prediction of vegetative bud burst timing (Chuine 2000, Chuine et al. 2003). These models are developed using phenological data series and meteorological data as input variables, assuming certain cause- effect relationships between the biological processes underlying bud burst and the driving environmental factors (Hänninen and Kramer 2007). Since the regulation of bud burst is only partially understood, several bud burst models based on different ecophysiological assumptions have been developed. Models of bud burst developed to understand the annual variation in plant phenology can largely be classified into two groups: theoretical and mechanistic models, according to the type of data the models are based on (Chuine et al. 2003).

Statistical models for bud burst are the simplest models, relating the timing of bud burst to environmental factors. The forcing effect of temperature on bud development and growth is considered to start from an arbitrary date in winter or early spring when dormancy is considered to be released. Bud burst is then assumed to occur when a certain number of accumulated heat units is reached. The relationship between temperature and growth assumed by the model may be linear, with a threshold (Arnold 1959), exponential (Hari et al. 1970) or sigmoid (Sarvas 1972, Caffarra et al. 2011b). Theoretical models of bud burst are more complex, including physiological assumptions about the relationships between the

plants and their environment, for instance the effect of chilling in dormancy release and the effect of temperature in promoting bud growth in the absence of dormancy. Dormancy release is assumed to occur when a certain number of accumulated dormancy breaking units or chilling units, is reached (chilling requirement).

Examples of mechanistic models are the Thermal Time model and the Alternating model (Cannell and Smith 1983). These models assume that for longer exposure to chilling, the rate at which bud development occurs increases when exposed to warm temperatures. The Alternating model also assumes a linear relationship between bud development and temperature, so that accumulated growth units are expressed as thermal time (day degrees > threshold temperature), and so that budburst date can be predicted by using only day degrees and chill days. The simple Thermal Time model has proved to be efficient in modelling chilling requirement for dormancy release (Hannerz et al. 2003). More recently developed models are based on the Alternating model, including more complicated functions in order to more accurately predict bud burst (e.g. Hänninen 1995, Chuine 2000 with a Unified model). However, the increase in accuracy is not always significant (Linkosalo et al. 2008). It is therefore important to expand existing models with only biologically significant parameters, such the influence of photoperiod on bud burst which was included into the Unified model to develop the Dormphot model (Caffarra et al. 2011a, 2011b).

Phenological models simulating the timing of bud burst and bud set are used for predicting increased risk of frost damage (Hänninen 1991), changes in the length of growing season and variation in species composition of forest ecosystems following global warming (Hänninen 1995; Chuine et al. 2003; Cleland et al. 2007). However, models based on quantitative effects of temperature on dormancy and bud burst have not always proved to be reliable in making predictions on a global scale. Nevertheless, they have shown a certain degree of accuracy in predicting bud-burst dates of different species on a local scale. For this reason, they could be successfully used on a local scale for predicting response of populations or clones to changes in environmental conditions.

1.3 Phenotypic plasticity in phenological traits in plants

1.3.1 *Phenotypic plasticity of forest trees and climate change*

Phenotypic plasticity is broadly defined as environmentally-induced phenotypic variation (Stearns 1989). It is widely known that individuals can modify their phenotype as a response to changes in the environment, and that these plastic responses may be adaptive (Pigliucci 2001). However, phenotypic plasticity is not easily quantified, and discussion exists on its role in adaptation to climate change (Gianoli and Valladares 2011).

With a rapidly changing climate that brings about rising average global temperatures, increasing frequency of extreme events, and 'new' pests and diseases, trees might not be optimally adapted to their environment any more, and the ultimate possibilities for the populations are (1) to adapt rapidly to the changing conditions, (2) to migrate to ecological niches that have become more suitable, or (3) to become extinct (Aitken et al. 2008). At present, phenotypic shifts in tree phenology are being observed which provides us with evidence that species and ecosystems are being influenced by global changes in the environment (Chmielewski and Rotzer 2001, Cleland et al. 2007). However, there is also evidence that rapid changes such as a growth decline in common beech (*Fagus sylvatica*) are occurring as a response to climate change (Jump et al. 2006), and some species ranges are already changing (Parmesan 2006). Hence, we can ask ourselves how far the phenotypic plasticity in phenology of plants can reach, what the possibilities are for plants to adapt genetically, and how drastic the consequences could be in terms of migration and extinction. The next section examines the issue of evolutionary and environmental constraints that are limiting factors in the plasticity of phenotypes.

1.3.2 *Constraints of phenological patterns in bud burst of forest trees*

While temperature and photoperiod are generally assumed to be the direct cues for bud burst, trees will not necessarily show exactly the same response to these cues. There is a certain

amount of plasticity in their response, and plasticity in phenological events is likely to be constrained by several factors, including plant morphology, physiology, and more generally the genetic and epigenetic background of each individual (Rathcke and Lacey 1985). The following section examines four of the possible constraints (mechanisms or processes that limit the ability of the phenotype to evolve; Schwenk and Wagner 2003) that may affect the timing of dormancy and bud burst in forest trees.

1.3.2.1 Resource limitation

Phenological processes require energy and thus may be influenced by resource availability and the plant's ability to absorb and allocate these resources (carbon, mineral nutrients, and water; Rathcke and Lacey 1985). However, different studies have given contradictory results. According to Roberntz (1999), bud burst in *Picea abies* (L.) H.Karst. was not affected by elevated CO₂ concentration and Sigurdsson (2001) found no effect of changes in nutrient status on bud burst in *Populus trichocarpa*. However, an advance in the timing of bud burst, caused by increased nutrient levels was demonstrated in *Picea sitchensis* (Murray et al. 1994) and in *Pinus sylvestris* L. as caused by elevated CO₂ levels (Jach and Ceulemans 1999). In contrast to these findings, Wielgolaski (2001) found indications that high nutrient levels can delay spring growth resumption in several woody species, and Murray et al. (1994) also observed a later flushing of *P. sitchensis* (Bong.) Carr. with elevated levels of CO₂ (in combination with low nutrient conditions). Water availability is also expected to have an effect on the timing of bud burst, mainly in Mediterranean areas, where trees may have to deal with periods of severe drought which can affect growth and the initiation of leaf primordia for several years after the drought period. But, according to Kramer et al. (2000), it is the foliage density (taking both the amount and size of leaves into account) that is affected by water limitation, rather than the timing of the phenophases. In Norway, precipitation was positively correlated with bud burst in *Betula pubescens* (Wielgolaski 2001). Because the moisture requirements of trees are very species-specific, the effects of water availability bud burst is very likely to vary among species (Nielsen and Jørgensen 2003).

1.3.2.2 Morphological constraints

Tree species that flush early in the season have xylem vessels with relatively narrow diameters. Their wood is diffuse-porous because the vessels have similar size throughout the growing season and tree rings are not very discernable (Figure 1.2 A). Most members of the genera *Populus* and *Betula* have diffuse-porous wood. Later-leafing trees are characterized by ring-porosity, with wider vessels in the earlywood and narrower vessels in the latewood (Figure 1.2 B), e.g. *Quercus* spp. and *Fraxinus* spp. (Lechowicz 1984).

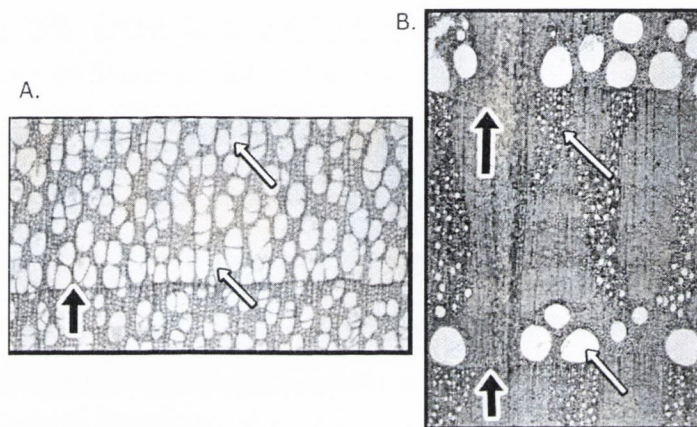


Figure 1.2 Photomicrographs of wood cross-sections from A. *Populus tremula* (diffuse-porous) and B. *Quercus petraea* (Mattuschka) Liebl. (ring-porous). The black arrows show the edge of a tree ring, in which the lower white arrows indicate the vessels formed in spring (earlywood) and upper white arrows indicate the vessels formed in the late summer (latewood). (Photos Hans Beeckman, InsideWood Database 2010)

Larger vessel diameters contribute to a greater hydraulic conductivity but are more vulnerable to embolism, caused by drought or by freezing and thawing cycles of the xylem sap under tension. If severe enough, embolism can limit growth. Diffuse-porous species are less vulnerable to embolism after winter frost than ring-porous species, but conifers (with only tracheids) are the most resistant (Sperry and Sullivan 1992). The correlation between the loss of hydraulic conductivity by late winter and the timing of bud burst in the spring has been demonstrated by Wang et al. (1992). Other morphological characteristics like crown architecture, foliage and branch structure, also influence phenological patterns (Ford 1992).

1.3.2.3 Avoidance of herbivory

Not only interspecific variation in phenology has been documented; even different individuals from the same species often can show asynchrony in bud burst in a given population (Wesolowski 2006). This is possibly a strategy to avoid herbivory, as it can have a negative effect on defoliation by insects. This was demonstrated in *Quercus* spp. populations by Mopper and Simberloff (1995), and by Tikkanen and Julkunen-Tiitto (2003). In tropical areas, where climatic fluctuations are less pronounced, herbivory (among other biotic factors) has been found to represent an important constraint on leaf phenology. The mechanisms suggested that reduce damage are (1) production of leaves when herbivore abundance is low, and (2) synchrony in flushes to saturate herbivores (Aide 1988).

1.3.2.4 Genetic and epigenetic constraints

Natural selection on overall fitness usually acts upon a whole suite of traits, rather than upon one individual trait (Geber and Griffin 2003). Therefore, genetic correlations among traits may prevent independent evolution of a specific trait (Etterson and Shaw 2001). Quantitative Trait Loci (QTL) mapping studies have supported the polygenetic nature of the phenotypic differences in traits affecting bud set and bud flush in *Populus trichocarpa* and *P. deltoides* (Bradshaw and Stettler 1995, Frewen et al. 2000). Although these two processes are separated in time, Frewen et al. (2000) found considerable correlations among QTL for bud flush and bud set, which may indicate a pleiotropic effect (when one single gene or allele controls the development of two or more traits), possibly resulting from sharing parts of a biochemical pathway. Other examples include indications for genetic correlations between bud burst and growth traits in *Quercus robur* L. (Scotti-Saintagne et al. 2004) and between bud phenology and frost hardiness in *Populus* hybrids (Howe et al. 2000).

Not only do genotype and environment contribute to phenotypic variation, but these two factors also interact. The molecular key to this interaction is epigenetics. The most important epigenetic effects, triggered by environmental factors are the methylation of DNA and post-

translational histone modifications, which cause changes in gene expression without any changes in genotype (Richards et al. 2010). Plants use these epigenetic mechanisms extensively to control development and as a basis for 'memory' of certain expression levels within and across generations (Henderson and Jacobsen 2007, Rohde and Junttila 2008). For instance, Sung and Amasino (2005) have demonstrated that a prolonged period of cold leads to histone modifications of loci involved in repression of flowering time locus C (*FLC*), an *Arabidopsis* flowering repressor that is discussed further in this chapter. Because of the variable heritability of epigenetic effects, evidence of local adaptation can be masked by or due to epigenetic effects (Rohde and Junttila 2008). For instance, Kvaalen and Johnsen (2008) found that in Norway spruce, the prevailing temperature during embryo development determines in each seed the timing of bud set of the future plant. This phenomenon is probably caused by epigenetic mechanisms generating variability within a population and also enabling a quick adaptation to changes in climate (Rohde and Junttila 2008). The existence of epigenetic effects in organisms is well accepted, however the techniques for quantifying the effects and the implications for ecology and evolution are still under discussion for this relatively new field of research (Richards et al. 2010).

1.3.3 Conclusions

Great advances have been made recently in the genomics of phenological and growth traits of trees, in order to identify genomic regions, genes, or polymorphisms that are associated with the control of these traits (Grattapaglia et al. 2009), so that predictions of climate change can take evolutionary change better into account. In addition to the constraints discussed above, the potential for adaptation of tree populations to climate change depends on the genetic variation within the populations, dispersal possibilities, and competition with other species (Antonovics 1976, Rathcke and Lacey 1985, Savolainen et al. 2007). Ultimately, it is the adaptive nature of plasticity that allows organisms to survive in different circumstances. Unfortunately, these adaptive values are difficult to assess, but considering the genetic variation observed in plasticity patterns for ecologically important traits, there is potential for the evolution of adaptive plasticity (Sultan 2000). This may be hopeful in future, as high

phenotypic plasticity will be valuable for plant species to be able to deal with the predicted increase in extreme weather events that comes with climate change (Jump and Peñuelas 2005).

1.4 Dormancy and bud burst of vegetative tree buds

Plants have developed the mechanism of dormancy in order to survive temporary unfavourable conditions by arresting growth and thus saving energy and vulnerable tissues during the harsh period. Examples of unfavourable conditions are a yearly recurring winter cold or drought, in which case dormancy is considered a phenological event (Crabbé 1994). Even though different plant structures can undergo dormancy (bulbs, tubers, seeds, buds etc.), the focus here is on bud dormancy in temperate, woody plants. The annual growth cycle of woody, perennial plants is composed of distinctive, but intergrading physiological phases, the dormancy phases (Figure 1.3). These phases are synchronised with the annual course of temperature at their natural growing sites (Hänninen and Kramer 2007). The classical definition of the term dormancy is ‘any case in which a tissue predisposed to elongate does not do so’ (Doorenbos 1953). In this traditional sense, it is only relevant to shoot apical meristems, but more recently, the term is also used to refer to a more general state of increased resistance to stress of the entire plant (winter hardening), or to the mitotic activity of cells or buds (Lavender 1985).

Table 1.1 The phases in an annual cycle of vegetative buds, and the mechanisms controlling each phase.

<i>Annual cycle phases</i>	<i>Control mechanism</i>
<i>1. Growth</i>	
a. Growth induction	Growth is induced under the influence of favourable temperature and photoperiod conditions. Growth resumption results in bud flush, shoot elongation and leaf unfolding.
b. Bud set	At the end of the growth phase, under the influence of temperature and photoperiod, growth cessation starts and new buds are formed.
<i>2. Dormancy</i>	
a. Paradormancy	Growth is inhibited by the buds. The inhibition is influenced by other organs or tissues.
b. Endodormancy	Growth is autonomously inhibited by the buds at a cellular level. Endodormancy is released under the influence of time and chilling temperature.
c. Ecodormancy	Buds are receptive to temperature and photoperiod changes. Growth is only inhibited while external conditions are not favourable.

As summarised in Table 1.1, the two major phases in the annual cycle are (1) a relatively short period of growth, shoot elongation and finally bud set, and (2) a longer period of dormancy during which there is no elongation, although lateral growth of the cambium can occur (Lavender 1985). During the dormant phase, a continuous transition occurs between (a) an inhibition of bud growth within the bud, but induced by other organs or tissues (paradormancy), (b) an inhibition within the bud that results in the inability of the organ to grow even when conditions are favourable (endodormancy or true dormancy), and (c) a restriction of growth within the bud caused by environmental factors (e.g. drought or cold temperatures) and perceived directly by the dormant tissue (ecodormancy or quiescence). The relative duration of these phases can differ among species and environments (Crabbé 1994). Figure 1.3 illustrates the annual cycle phases in a continuous transition throughout the seasons, and the visible changes on a tree. After growth cessation and bud set in late summer, the buds go into a paradormancy phase. Endodormancy is induced in late autumn. Senescence and hardiness are the results of dormancy. Finally, just before dormancy release in spring, the buds go into an ecodormancy phase in which they are receptive to favourable environmental conditions. Flowering, bud burst, leaf expansion and wood formation are examples of visible signs of growth-reactivation. Figure 1.3 illustrates well that all phases are in a continuous transition and have no definite beginning or ending.

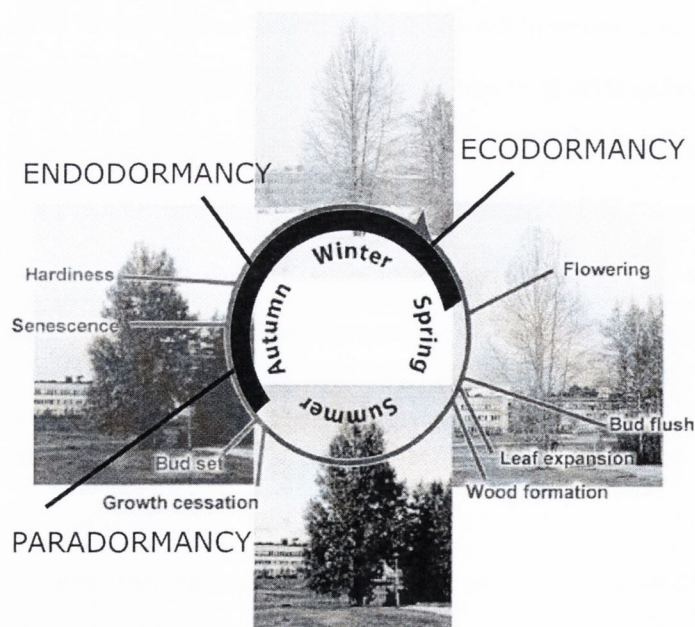


Figure 1.3 The annual cycle of a *Populus* tree. The dormancy phases are indicated by the black half-circle. (after Jansson and Douglas 2007).

Bud dormancy is induced by short day lengths and cold temperatures in order to protect the vulnerable meristems. Bud break however, is mainly controlled by the cumulative sum of temperatures to which buds are exposed after a required cold period (Lang et al. 1987). It has to be kept in mind that these external triggers do not directly affect the changes within the buds: during the dormancy cycle, buds undergo physiological stages that change their sensitivity to certain hormones, which are activated or deactivated (Crabbé 1994). This relationship with temperature illustrates the dependency of plants on their environment and the importance to be finely tuned, or adapted, to their natural conditions.

1.5 Genetic control of dormancy-related traits in trees

1.5.1 *Local adaptation of trees in phenological traits*

It is well known that trees respond to their environment by adjusting the timing of dormancy phases through the mechanism of phenotypic plasticity, but whether the response is either inherent to the plant (locally adapted) or merely triggered by the environmental conditions *in situ* is difficult to tell. A method for testing local adaptation is a common garden experiment where diverse ecotypes are grown at one location, and with the environmental conditions of the site taken into account (Rutter and Fenster 2007). Common garden experiments provide evidence that phenological events in trees are genetically controlled. For example, when *Populus* spp. clones originating from different latitudes were grown at the same location the clones from lower latitudes flushed earlier than the ones from higher latitudes (Pellis et al. 2003). However, Vitasse et al. (2010) found a high amount of phenotypic plasticity in a common garden experiment along an altitudinal gradient, using common beech and oak. Furthermore, Bradshaw and Stettler (1995) calculated that up to 98% of the total phenotypic variance of bud burst in *Populus* hybrids was explained by heritability, although plasticity was not quantified. Adaptive population differentiation (the possibility to differentiate populations based on the variation within a single gene or gene region) in temperate and boreal forests has occurred despite substantial gene flow levels (Ingvarsson et al. 2006). Seeing the importance of local adaptation, the biotic and abiotic influences surrounding plants define the distribution of species and biomes (Norby and Luo 2004). However, although they are well

adapted to their environment, forest trees can also survive and grow outside their natural range (Savolainen et al. 2007). In addition, different co-occurring tree species can also show considerable inter-specific variation in their responses to the environment they are adapted to (Lechowics 1984; Ogaya and Peñuelas 2007). It has to be kept in mind that plasticity and genetic control for a particular trait do not rule each other out, not in the least because plasticity itself is an adaptive trait (Pigliucci 2001).

As global warming is expected to have major impacts on plant functions and fitness through changes in phenological events, the modelling of these events has become a useful tool for predicting species range under future climate scenarios (Chaine and Beaubien 2001) and carbon production of forests (Lieth 1971). Since genetic variation among populations is an indication for the possibility of the populations to adapt to new environmental conditions, the genetic variation in phenological traits is very relevant for these models (Chaine et al. 1999). The selective pressures working on the phenology of a tree - a component of reproductive success and thus of its survival - are climate-dependent. Several studies have shown that rapid evolution in response to climate change may be widespread in natural populations, based on genetic variation already present within the population (Jump et al. 2006, 2007). For example, Jump et al. (2006) have found that gene frequency varies predictably with temperature at a *Fagus sylvatica* locus identified as being under selection in Spain. Furthermore, the probability of presence of the dominant marker allele declines over the latter half of the 20th century, correlated with rising temperatures in the region of the beech population (Jump et al. 2006).

1.5.2 Ecological significance of local adaptation of phenological traits

Populations of different tree species across Europe demonstrate the evolutionary response of plants to climatic gradients. For example, species such as *P. tremula* are distributed from the Mediterranean to the Subarctic and across a broad longitudinal range from the European Atlantic coast inland to Russia. These species are adapted to the local conditions of growth as a result of an interaction between physical limits and competition (Savolainen et al. 2007). Local adaptation to climate has been well documented in numerous tree species. They may

be capable of surviving outside their normal range but are prevented from doing so by competition from other species (Aitken et al. 2008). This is clearly demonstrated by the fact that a species from a particular climatic zone is able to grow when placed in a series of botanic gardens across a diverse range of environmental conditions throughout the continents. A common garden experiment on pine bud set across a latitudinal gradient from Spain to Finland showed that the timing of bud set was determined by the origin of the plant rather than the conditions of growth (García-Gil et al. 2003). It has also been confirmed in other studies that phenology related traits such as bud set and budburst are strongly subjected to natural selection (Yakovlev et al. 2006). However, Vitasse et al. (2009, 2010) have shown in several temperate tree species along an altitudinal cline that spring phenology has both a high magnitude of plasticity and a high level of heritability.

It has been shown that rapid responses to climate change are already widespread in natural populations of plants and tree species (Parmesan 2006, Savolainen et al. 2007, Jump et al. 2008). However, the genetic basis of this response is still unclear. And although the ecophysiology of several phenological phases has been studied extensively in plants, knowledge of the molecular mechanisms behind them is still limited (Ruttink et al. 2007). Until recently, it was commonly believed that the transition between dormancy and growth was solely a result of the balance between promoting and inhibiting hormones. However, functional genomics and other new technologies have showed that the control of dormancy is more complicated and works on different levels (Yanovsky and Kay 2002, Arora 2003, Bohlenius et al. 2006, Rohde et al. 2007, Ruttink et al. 2007).

1.5.3 Candidate genes for the control of dormancy and bud burst

1.5.3.1 End-of-season phenology

Most genetic studies involving phenology cover phases towards the end of the growing season such as bud set, growth cessation and induction of dormancy (e.g. Rohde and Boerjan 2001, Savolainen et al. 2004, Ruttink et al. 2007). Since red and far-red light are detected by proteins called phytochromes in plants, the genes for these photoreceptor

proteins are putatively involved in end-of-season phenology (Howe et al. 1996). Individual phytochromes can have different functions in the cell and some of the phytochrome genes have overlapping functions. For instance, *phyB2* has been linked with bud set as well as bud flush in *Populus* (Smith 2000). *PhyA* plays a role in growth cessation, through the regulation of *FT* (FLOWERING LOCUS T) and *CO* (CONSTANS) transcription factors (Yanovsky and Kay 2002). Olsen et al. (1997) demonstrated that transgenic aspens over-expressing the Oat *phyA* gene initiated growth cessation only in response to very short photoperiods of 6 h, as opposed to the usual 14-16 hours. The amount of *phyA* gene expression by the plant might thus affect photoperiodic responses in trees (Olsen et al. 1997). Ruttink et al. (2007) applied metabolite and transcript profiling to bud samples taken at weekly intervals during the time course from short day-induced dormancy induction to endodormancy in *Populus tremula* x *Populus alba* L.. Their results showed that light, ethylene, and abscissic acid transduction pathways consecutively controlled the transition from bud formation to acclimation and to dormancy. However, while considerable molecular and biochemical changes occurred in the first few weeks of SDs (short days), changes in gene expression were not significant during the time of transition to endodormancy, suggesting a different control for its fixation (Ruttink et al. 2007).

Recent findings showed that in *P. tremula* the response to SDs was mediated by orthologues to the genes *CONSTANS* (*CO*) and *FLOWERING LOCUS* (*FT*) in *Arabidopsis* (Böhlenius et al. 2006). Transcript profiles from aspen grown with different critical photoperiods showed differences in the diurnal oscillation patterns of the expression of *PtCO*. The importance of this finding was supported by the fact that the diurnal phase of *CO* expression controls *FT* (affecting growth and flowering) in *Arabidopsis*. In addition, SD-insensitive transgenic trees did not show any repression in *PtFT* (*Populus trichocarpa* *FLOWERING LOCUS T*) expression. Böhlenius et al. (2006) supported the view that the transition between LD (Long Days) and SD was perceived by the plant as the moment when the expression of *PtCO* (*Populus trichocarpa* *CONSTANS*) peaks in darkness. This, in turn, triggered a downregulation of *PtFT*, and induced growth cessation (Böhlenius et al. 2006). More evidence from different plant species argued for a regulation of growth and floral transition by *FT* in potato (Rodriguez-Falcon et al. 2006) and poplar (Hsu et al. 2006).

Little is known about the changes occurring in connection with endodormancy establishment. This particular phase is the final step in a series of transformations, which are difficult to separate. Ruttink et al. (2007) showed that photoperiod started a signaling cascade, which probably triggered subsequent molecular events and activated different pathways. In an extensive survey, Rohde et al. (2007), examined gene expression during the induction, maintenance and release of dormancy in poplar (*P. tremula* x *P. alba*), and found a global change in expression patterns after 24 SDs, in accordance with previous findings (Ruttink et al. 2007). It occurred concurrently with bud set and was thus related to morphological changes in apical bud morphology (Rohde et al. 2007). The transition to endodormancy was not marked by dramatic changes in gene expression, but a cluster of novel candidate genes were proposed for functions during chilling requirement. One of these is a DNA-binding protein with linker-histone domains that has a potential regulatory role in dormancy release (Rohde et al. 2007).

1.5.3.2 Beginning-of-season phenology

Few genetic studies have focused solely on spring phenological events for perennial plants, and many genes appear to be involved in both onset of growth as well as growth cessation, e.g. *phyB2*. The annual herbaceous plant *Arabidopsis thaliana* has been studied extensively with regards to flowering. Therefore, many homologous and orthologous *Arabidopsis* genes are studied in trees (Horvath 2009).

The control of endodormancy release is still a matter of speculation. The similarities between chilling fulfilment and vernalization might suggest a role of genes orthologous to *FLC* (*Flowering Locus C*), whose repression in *Arabidopsis* occurs after exposure to cold temperatures (Sung and Amasino 2005). Indeed, *FLC*-like genes have been found to be differentially expressed during dormancy release in poplar (Coleman and Chen 2008).

Similar to endodormancy fixation, dormancy release is also characterized by the expression of DNA-binding proteins (Yakovlev et al. 2006). This, and additional evidence for DNA

methylation observed in potato buds (Law and Suttle 2003), suggests that epigenetic changes might play an important role in dormancy (Horvath et al. 2003).

1.6 Study species

1.6.1 Species selected

The species chosen for this study were the widespread European tree species *Populus tremula* L. and *Betula pubescens* Ehrh. The selection of these species was made on the basis of their presence in the IPG network, the status as a model species for *P. tremula*, and the previous studies carried out in Trinity College Dublin on dormancy in *B. pubescens* (Caffarra and Donnelly 2010, Caffarra et al. 2011a, 2011b).

1.6.2 European aspen (*Populus tremula* L.)

1.6.2.1 Taxonomy

Populus tremula (European aspen, common aspen or Eurasian aspen) was described as a species by Linneaus in 1753 in his *Species Plantarum*, and belongs to the genus *Populus* within the Salicaceae or Willow family. The Salicaceae Mirb. are placed in the order Malpighiales Mart. together with 38 other families, and are considered to be related to the Passifloraceae (Passionflower family) and Violaceae (Violet family), although the relationships between the families in the Malpighiales are still poorly understood (Chase et al. 2002). Salicaceae is a near cosmopolitan family (Figure 1.4) of trees and shrubs characterized by distinctive glandular leaf teeth ('salicoid teeth'). Previously the family contained only 3 genera, but was expanded by the Angiosperm Phylogeny Group with several members of the Flacourtiaceae to 55 genera (APG II 2003).

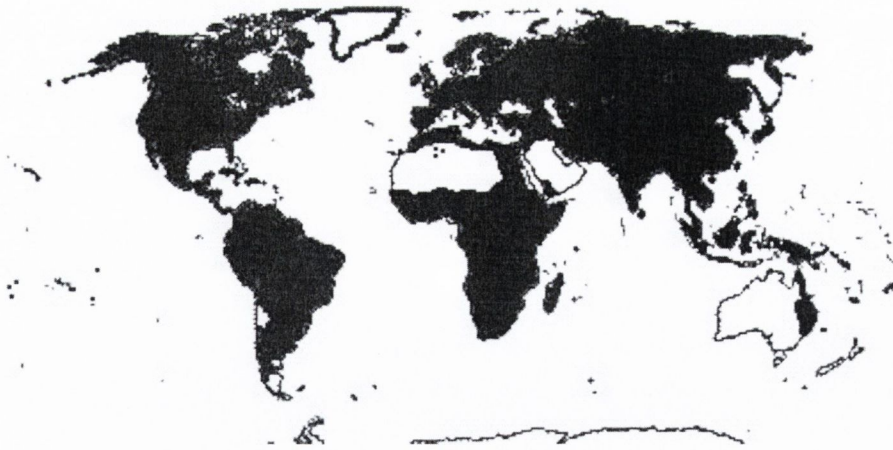


Figure 1.4 Distrubution of Salicaceae in black (Stevens 2001 onwards).

Populus is a genus of deciduous trees (rarely semi-evergreen), commonly known as aspen, cottonwood or poplar. It has a wide natural distribution in the Northern Hemisphere and a small representation in tropical Africa (Bradshaw et al. 2000). Although phylogenetic analyses show that *Populus* is a clearly defined, monophyletic sister group of *Salix* (willows), extensive interspecific hybridisation causes some controversy about the number of species and classification within the genus. For instance, *P. tomentosa* x was described as a separate species in 1867 by Carrière, but only identified as a natural hybrid by Eckenwalder in 1996. Eckenwalder (1996) recognises 29 species, divided into six sections (between which theoretically no hybridisation occurs) based on morphological and ecological differences (Table 1.2). Although recent molecular studies have largely supported this classification, certain polyphyletic relationships have been discovered due to past hybridisation between the sections, and the placement of several species remains controversial (for instance *P. nigra* L., Hamzeh and Dayanandan 2004). *Populus tremula* is classified in the section *Populus* syn. *Leuce* Duby (Table 1.2), subsection *Trepidae* Dode.

Table 1.2 Most recent classification of *Populus* by Eckenwalder (1996), s.l. (*sensu lato*): indicates that other species which are often recognized as distinct in the literature are retained as subspecies, or other

Section					
Abaso	Turanga	Leucoides	Aigeiros	Tacamahaca	Populus
Mexican poplars	Subtropical poplars	Necklace poplars	Cottonwoods	Balsam poplars	Aspens
<i>P. mexicana</i>	<i>P. euphratica</i>	<i>P. glauca</i>	<i>P. deltoides</i>	<i>P. angustifolia</i>	<i>P. adenopoda</i>
	<i>P. ilicifolia</i>	<i>P. heterophylla</i>	<i>P. fremontii</i>	<i>P. balsamifera</i>	<i>P. alba</i>
	<i>P. pruinosa</i>	<i>P. lasiocarpa</i>	<i>P. nigra s.l.</i>	<i>P. ciliata</i>	<i>P. gamblei</i>
				<i>P. laurifolia</i>	<i>P. grandidentata</i>
				<i>P. simonii s.l.</i>	<i>P. guzmanantlensis</i>
				<i>P. suaveolensis</i>	<i>P. monticola</i>
				<i>P. szechuanica</i>	<i>P. sieboldii</i>
				<i>P. trichocarpa</i>	<i>P. simaroa</i>
				<i>P. yunnanensis</i>	<i>P. tremula s.l.</i>
					<i>P. tremuloides</i>

species or varieties are included.

1.6.2.2 Distribution, morphology and ecology

Distribution and occurrence

European aspen has a large natural distribution in cool temperate, oceanic and boreal areas of Asia and Europe (Figure 1.5). In the south of its range, it occurs at higher altitudes (Rushforth 1999). Aspen grows where annual precipitation exceeds evapotranspiration and is very frost hardy, shown by its ability to grow in the harsh conditions near the tree line. It favours moist, well-aerated soils and is able to grow in a wide variety of soils ranging from shallow and rocky to deep loamy sands and heavy clays (von Wühlisch 2009). In Ireland and Britain, it is a widespread but sporadically occurring species in native woodlands, although more frequent in Britain than in Ireland (Worrell 1993).

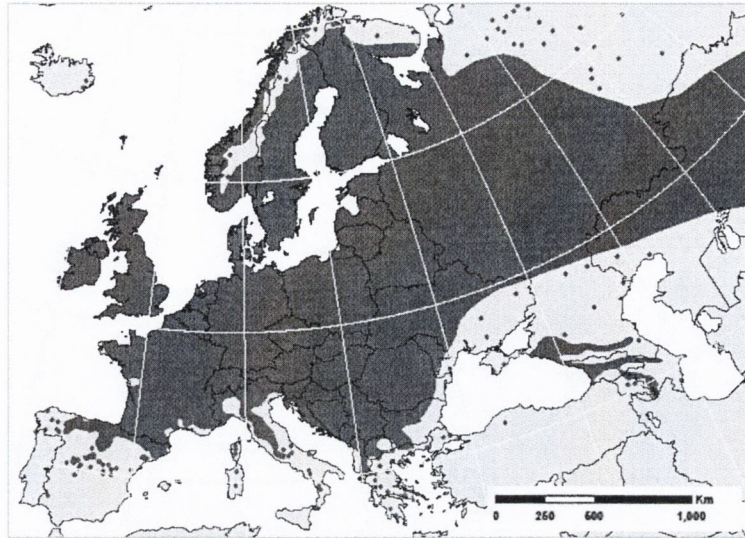


Figure 1.5 Distribution of *Populus tremula* in Europe in black (EUFORGEN 2009)

Morphology and habit

Populus tremula is a deciduous tree growing to 40 m in height. The bark is pale and smooth on young trees, becoming grey and rugged on older trees. The adult leaves are nearly round (Figure 1.6), 2–8 cm in diameter, with big wave-shaped teeth and a laterally flattened petiole, which allows them to tremble in even slight breezes, hence its scientific name. The leaves on seedlings and fast-growing stems of root sprouts are heart-shaped and often much larger. The flowers are wind-pollinated catkins (Figure 1.6) produced in early spring before the new leaves appear. The species is dioecious, with male and female catkins on different trees and the seeds are small and cotton-tufted (Johnson and More 2004, von Wühlisch 2009).

European aspen is often a principal coloniser, either by seeding or root suckers. It is capable of rapid growth under conditions with plenty of light and water. Root suckers are produced abundantly on the shallow lateral roots after an individual has been damaged or destroyed, e.g. by cutting, fire or diseases leaving an open space exposed to sunlight (Worrell 1993, Bradshaw et al. 2000).



Figure 1.6 *Populus tremula* twig with leaves (1), young twig with leaves and catkin (2), and root sprout (3). Plate from Flora Batava(1872), www.BioLib.de.

Ecology

Aspen trees have an important ecological value. They provide habitats for a wide variety of mammals and birds, including endangered species such as many woodpecker species (Worrell 1993). Numerous leaf, bark and wood inhabiting insects and fungi exist on aspen, among them *Melasoma* sp. an ectoparasitic mite feeding on the leaves, *Saperda* sp., boring the trunk, and fungi like *Phellinus tremulae* (Bondartsev) Bondartsev and P.N. Borisov, causing heart rot. They are also important food plants for the larvae of a large number of Lepidoptera species (Worrell 1993)

1.6.2.3 Importance and uses

Wood

The wood is diffuse-porous and relatively strong for its weight. Because of these features and its rapid growth, aspen wood is cultivated worldwide in plantations. It is used for pulp and paper, plywood, packing material, and it plays an important role in production of wood as a

renewable energy source (Bradshaw et al. 2000). Its woody biomass has good combustion properties compared to non-wood solid biofuels and fossil solid fuels (Kauter et al. 2003).

Trees

Other recent uses are in phytoremediation of environmental pollution in riparian zones (Dix et al. 2007) and as bioindicators of ozone pollution (Karnosky et al. 2003). Also in landscaping the European aspen is popular in many countries due to its unique shape and rustling noise of the leaves (Worrall 1995).

Propagation

Vegetative propagation of aspen is relatively straightforward, but only successful when using root suckers, root cuttings, and softwood cuttings taken from young plants. Hardwood cuttings will not easily be induced to grow (Worrall 1995).

1.6.3 *Downy birch (Betula pubescens Ehrh.)*

1.6.3.1 Taxonomy

Ireland has two native species of birch, *Betula pubescens* Ehrh. (Downy birch) and *Betula pendula* Roth. (silver birch), of which *B. pubescens* is the most common (Elwes and Augustine 1906). Birch species belong to the genus *Betula* in the family Betulaceae. *B. pubescens* was first described by German botanist Jakob Friedrich Ehrhart in 1791 (IPNI). The Betulaceae are a family of deciduous trees or shrubs, that are mainly occurring in the northern hemisphere (Figure 1.7) and are included in the order of the Fagales Engler.

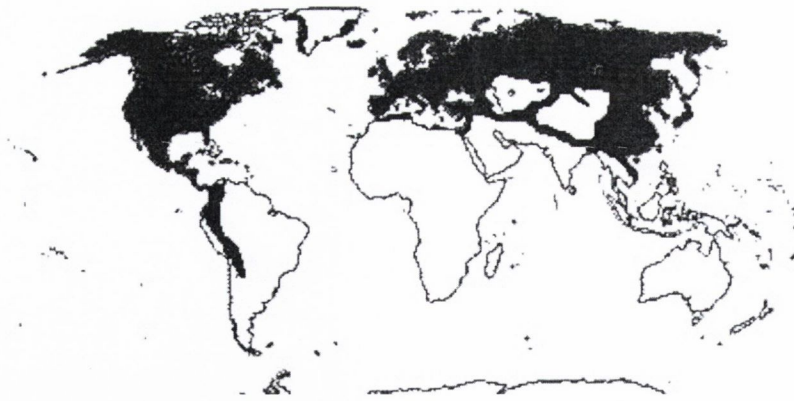


Figure 1.7 Distribution of Betulaceae in black (Stevens 2001 onwards).

The Fagales are a relatively well-resolved group of seven families: the Betulaceae, the Casuarinaceae, the Fagaceae (beech family), the Juglandaceae (walnut family), the Myricaceae, the Nothofagaceae (southern beeches), and the Ticodendraceae. Betulaceae are most closely related to Ticodendraceae, which are native in Central America, and Casuarinaceae, a tropical American family (APG III 2009). The family Betulaceae consists of two subfamilies and six genera with many well-known tree species: the genera *Alnus* (alder) and *Betula* (birch) are placed together in the subfamily Betuloideae, while *Carpinus* (hornbeam), *Corylus* (hazel), *Ostrya* (hop-hornbeam) and *Ostryopsis* form the Coryloideae (APG II 2003). Until 2003, the two subfamilies were considered to be separate families (Betulaceae and Corylaceae). The genus *Betula* is divided into five subgenera and contains 30 to 60 species, depending on the source (Stevens 2001 onwards)

1.6.3.2 Distribution, morphology and ecology

B. pubescens is a deciduous tree with creamy brown coloured bark. The young twigs are coated with a fine downy fur, hence the common and scientific name of this species. Its roots are quite shallow and it reaches an average height of 15-20m. The adult leaves are heart- or triangular shaped, with small teeth. The male flowers of the dioecious birch trees are wind-pollinated catkins that can be up to 6cm long. Birch is a pioneer species and will grow on poor soils. It can tolerate high elevations and wet soils quite well (Johnson and More 2004). Its natural range is similar to that of *P. tremula* and covers most of middle and northern Europe (Figure 1.8).



Figure 1.8 Distribution of *Betula pubescens* in Europe (dots) (Jalas and Suominen 1988)

6.3.3 Importance and uses

Birch has several human uses. The timber has traditionally been used in Ireland and the UK in houses, furniture, carts, ploughs, gates and fences. Also today, birch timber has a variety of uses, for instance in the paper industry because of its short fibers, and for plywood (Woodland Trust 2011). The bark was historically used for tanning leather and the sap was thought to have medicinal benefits for kidney stones and other ailments (Warren 2006). Birch is also used in modern medicine. For instance, the bark compound betulinic acid from *B. pubescens* has been found to reduce the growth of melanoma cancer cells (Pisha et al. 1995). The sap was also useful as it was considered to be a good source of sugar and was brewed into beer or wine, which is still a practice in Scotland. Birch trees are also still used frequently as an ornamental tree in gardens and in towns in Europe (Warren 2006).

1.7. Aims of this study

The main aims of this work were to explore the various mechanisms behind the vegetative spring phenological features in European trees, mainly dormancy release and bud burst, through three different approaches.

- (1) From an experimental approach (Chapter 2), the aims were to:
 - a. quantify the effects of chilling duration, forcing temperature, and photoperiod on the percentage and timing of bud burst in *P. tremula* and *B. pubescens* using controlled environment experiments and statistical methods
 - b. provide information from the controlled environment results to models predicting bud burst with future climate change
- (2) The modelling element of this study (Chapter 3) had the aim to:
 - a. find an explanation for the discovered effects of experimental conditions on the timing of bud burst using distribution curves of bud burst data and analysing the distribution parameters
 - b. obtain a mathematical relationship describing the response of timing of bud burst to the different conditions used in the experimental study presented in Chapter 2
- (3) On a molecular level (Chapter 4), the aims were to:
 - a. identify genes involved in dormancy release in *P. tremula* and obtain the sequences of the dormancy-related genes identified in natural European populations of *P. tremula*
 - b. quantify the genetic variation between and within the natural populations
 - c. detect indications for relationships between genetic variation and natural clines (latitudinal or longitudinal gradients; 'clinal variation')

The hypotheses tested were:

- (1) that a longer photoperiod, a longer chilling period and a higher forcing temperature decreased the timing of bud burst and increased the total percentage of bud burst in both species studied;

- (2) that despite the similar trends, *B. pubescens* and *P. tremula* show a different response to the experimental conditions, i.e. that they vary in chilling requirements and rate of bud burst;
- (3) that the distribution curves for bud burst provide insights in the progress of bud burst on a tree-to-tree scale, which is useful for modellers;
- (4) that the experimentally obtained data for bud burst under different conditions provide a sufficient basis for fitting a basic mechanistic phenological model;
- (5) that significant genetic variation was present in dormancy-related traits between natural populations of an outcrossing species such as *P. tremula*;
- (6) and that the genetic variation found between populations was correlated with a natural longitudinal or latitudinal cline.

The following three chapters presented here describe the threefold study on bud dormancy in *Populus tremula* and *Betula pubescens*. The study is divided into (Chapter 2) an experimental study of the relationship between the timing of bud burst and the environmental factors temperature and photoperiod in both species, (Chapter 3) a modelling study that was based on the results obtained in the experimental section, and (Chapter 4) an analysis of the genetic variation in dormancy-related genes focused only on the species *P. tremula*. Each of the following three chapters start with a short introduction to the study presented. Subsequently, a brief explanation is given of the methods used: the experimental design and statistical methods in Chapter 2 and Chapter 3, and the laboratory techniques and analysis methods in Chapter 4. In each of the chapters, the findings are described and demonstrated in the results section, after which a discussion section clarifies the results by comparing with previous studies, discussing the implications and suggesting directions for future research. Finally, Chapter 5 synthesises the general conclusions of this study, placed into a broader context. References are listed after each chapter rather than at the end for ease of reading.

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Chapter 2:

Environmental manipulations of the timing of bud burst in *Betula pubescens* Ehrh. and *Populus tremula* L. clones

2.1 INTRODUCTION

Bud dormancy is an essential part of the phenology of trees in temperate regions, where survival depends on the ability to cease and resume growth according to seasonal environmental variations. But rather than a uniform state of inhibited growth, dormancy is a gradual transition of different physiological conditions, named (1) paradormancy, (2) endodormancy, and (3) ecodormancy (Lang 1987, Arora et al. 2003). These conditions are respectively (1) an inhibition of growth in the buds induced by plant tissues outside the buds, (2) an inhibition from within the bud, and (3) a restriction of growth controlled by the environment (Arora et al. 2003). For most tree species, dormancy is induced mainly by short photoperiods during late summer and fall (Håbjørg 1972, Heide 1974), while the breaking of (endo-) dormancy is mainly triggered by winter chilling temperatures (Sarvas 1972, Champagnat 1993). Insufficient chilling during warm winters causes the endo-dormancy phase to extend and decrease more slowly (Sarvas 1972, Cannell and Smith 1983). The concept of chilling is not clearly defined, as each species and even ecotypes within a species can differ in their chilling requirements (e.g. Myking and Heide 1995, Li et al. 2003). It is usually expressed as the sum of hours or days below a certain threshold temperature, typically 10°C or 12°C, and above 0°C (Battey 2000, Körner 2006). After the breaking of endo-dormancy, plants enter the eco-dormancy phase as they become receptive to warm spring temperatures to resume metabolism and growth activity. Also the heat sum requirements (or thermal time) to release eco-dormancy vary between species, and there is a negative interaction between the level of chilling and the heat sum needed for bud burst: with more chilling, less heat is needed for flushing (Heide 1993). Besides temperature, photoperiod also plays a role in dormancy release, especially in higher latitudes where the need for a long photoperiod for bud burst to occur prevents a temperature-induced break of dormancy too early in the season (Körner 2006).

Overall, bud development in temperate trees is controlled by a combination of (1) chilling (temperature and duration), (2) photoperiod and (3) spring- or forcing temperatures. Although there is an extensive literature available on the effects of temperature and photoperiod on plants (see Chapter 1), the interactions between these factors and the differences between (and within) species require further clarification which makes predictions of phenology responses to a warmer climate challenging. Furthermore, the interactions between photoperiod and temperature are not fixed, meaning that particularly warm temperatures can partially override photoperiod controls, and particularly long days can partially override chilling requirements (Heide 1993, Caffarra et al. 2011a). Since the timing of phenological events has an effect on survival and reproduction of trees, selective pressures are at play and the responses measured are ultimately the results of adaptation to particular environments. Different species are therefore to be expected to have different phenological responses, which are important to understand for estimating the impacts of climate change at an ecosystem level. However most experimental studies focus on a single species (e.g. Håbjørg 1972, Heide 1993, Li et al. 2003). Here, bud burst is investigated in two native European and native Irish species, *Populus tremula* (European aspen) and *Betula pubescens* (downy birch).

Bud burst is often used as a measure of dormancy release, even though it is only one of the results of the breaking of dormancy. Gene functions and cell metabolism are reactivated before bud burst becomes apparent (Arora et al. 2003). Physiological signals such as hormone and sugar levels induce invisible processes within the plant such as cambium growth in the stem and meristem development within the buds even before bud elongation becomes visible (Horvath et al. 2003). Bud burst is however the most clear and observable phenological stage in wind-pollinated tree species such as birch and aspen, as the opening of the minute flowers arranged in catkins is not always clearly visible from ground level when observed on adult trees. Therefore, and because of its indicator value for climate change (Menzel et al. 2006) bud burst is a key phenophase that is monitored in Ireland's National Phenology Network and in the International Phenological Gardens (IPG) network. It is however not only important to monitor bud burst over a long term, but also to understand the mechanisms behind this phenological event.

Aims of this study

This study follows on from the work performed by Caffarra and Donnelly (2010) and Caffarra et al. (2011a, 2011b) who investigated the effects of forcing temperature and photoperiod on the rates of dormancy release and induction in *B. pubescens*. The findings from these studies revealed that (1) photoperiod during chilling has no effect on the timing of bud burst, (2) longer exposure to chilling increased growth rate and decreased the time to bud burst, and (3) bud burst timing was advanced by a photoperiod above a critical threshold. The results of these experiments with mainly a focus on the forcing effect, suggested that more research was needed on chilling effects, and a possible interaction effect between chilling and photoperiod.

The aim of this study is to quantify the primary effects and interactions of temperature (in the form of chilling) and photoperiod on the percentage and timing of bud burst in both *B. pubescens* and *P. tremula*, using controlled environment experiments. The results will form part of the framework for use in process-based phenological models. Furthermore, the results will have implications for forestry and conservation, because as winter temperatures become milder due to climate change, critical chilling thresholds might therefore not be reached in the future.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

All *Betula pubescens* (birch) and *Populus tremula* (aspen) plants used in the experiment were vegetatively propagated so that differences in the responses of the plants could only be attributed to varying environmental conditions and thereby any genotypical differences could be excluded.

B. pubescens

For propagation purposes, 280 cuttings of same year growth were taken in the last week of November 2008 from adult birch trees growing at the John F Kennedy Arboretum (New Ross, Co. Wexford, Ireland). The *B. pubescens* trees used were clones originating from the International Phenological Gardens (IPG) network, in order to follow on from previous phenology studies using the same genotype. The IPG network was established in 1959 to collect phenological data from sites across Europe (Chmielewski and Rötzer 2001). Genetically identical trees and shrubs were planted in all of these gardens in order to assess the timing of phenological events in trees on a wide geographical range. The birch clone used was originally from Germany and was vegetatively propagated for the purposes of this work, as described by Hartmann et al. (1997). After the cuttings were taken, they were immediately planted for propagation in Teagasc Horticultural Centre (Kinsealy, Co. Dublin) where they were treated with Seradix rooting powder and planted in 10 cm-deep trays on heated benches. Subsequently, they were covered with transparent plastic to avoid dehydration. However, the cuttings did not survive and only yielded 20 viable plants, which might have been due to the relatively late date in the season when the cuttings were taken or the transport duration between the two sites. Subsequently, another 240 birch cuttings were taken in July 2009, this time from juvenile plants propagated in 2003 and 2004 from the same adult trees in the John F Kennedy Arboretum. Propagation of these trees is described in Caffarra and Donnelly (2010). The cuttings were transferred to a mist unit in Teagasc Horticultural Centre in Kinsealy to root (Figure 2.1). It was expected that these actions would have given a higher success rate, but again not enough cuttings survived in order to perform the experiments. Possible

factors in the second propagation failure were the mist unit to work insufficiently in keeping the twigs moist or a high amount of direct sunlight also causing dehydration. The birches eventually used in the experiments were the juvenile trees (height between 30 and 45 cm) that were propagated in 2003 and 2004 (Caffarra and Donnelly 2010) and kept outdoors in 1l plastic pots at the Trinity College Botanic Gardens, Dublin and subsequently at Teagasc Horticultural Centre in Kinsealy.

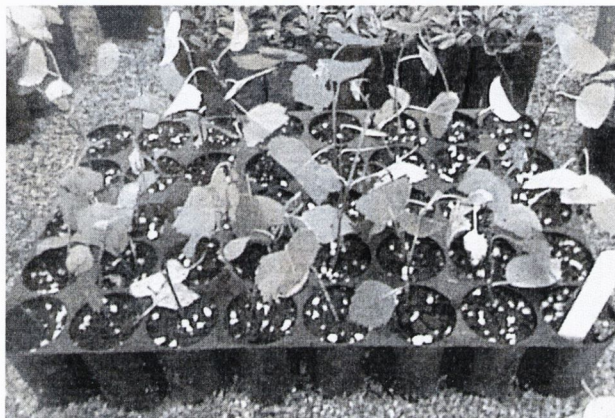


Figure 2.1 *B. pubescens* cuttings in misting unit of Kinsealy Research Centre one week after planting (July 2009)

P. tremula

The aspen trees were commercially cloned juvenile trees purchased from Cappagh nurseries (Aughrim, Co. Wicklow, Ireland; EU Plant Passport 7238). The clones were propagated from root cuttings and were grown outdoors at the site of the nursery. For the experiments, the 3 to 4-year old trees with heights between 80 and 120 cm were transplanted into plastic pots with a diameter of 15-20 cm containing John Innes No. 3 compost, and were kept at Teagasc Horticultural Centre in Kinsealy.

There was no overlap in plants being used in the following experiments; all trees used were exposed to natural temperature and photoperiod conditions in the year prior to commencement of the experiments. For the second year's experiments, aspen trees of the same age and size as the previous year were also purchased from Cappagh nurseries in Wicklow, and the birch trees used were from the same propagation batches as before. The

birch trees were therefore one year older. The average viable number of buds per aspen tree was 59, ranging between 24 and 152 buds per tree, and the average number of buds for birch trees was 16 (between 4 and 41 buds per tree).

2.2.2 Experimental design

EXPERIMENT 1

In the autumn and winter of 2009-2010, experiments investigating dormancy release were conducted on 30 3-4 year old aspen trees and 30 5-6 year old birch trees to determine the effects of photoperiod and chilling requirement on the timing and percentage of budburst. The experiments consisted of two phases.

The first phase started on 25th November 2009, with 15 trees of each species. The trees were kept outdoors at Teagasc Horticultural Centre, Kinsealy since August 2009, to receive natural chilling in ambient conditions. On the day of transfer into the growth chambers, the trees had only partially fulfilled their chilling requirements: they had undergone 35 chilling days (number of days with average temperature $\leq 10^{\circ}\text{C}$ since 1st September, which is a rough estimation of the amount of chilling; Myking and Heide 1995). Three different photoperiod conditions (8, 12 and 16 hours of daylight) were created in a growth chamber (Convion PGR15, Trinity College Dublin, Figure 2.2).



Figure 2.2 Convion PGR15 growth chamber, one of the growth cabinets used for environmental control of *P. tremula* and *B. pubescens* clones.

The lights in the growth chamber were switched off automatically at night between 1 and 9am. Five trees from each species were subjected to the different photoperiod lengths by covering them with non-transparent black plastic at different times of day: 10 trees (5 aspen and 5 birch trees) were covered at 5pm resulting in an 8-hour photoperiod, another 10 trees (5 aspen and 5 birch trees) were covered at 9pm resulting in a 12-hour photoperiod and the remaining trees remained uncovered resulting in a 16-hour photoperiod treatment. The temperature in the growth chamber was kept constant at 10°C, a temperature that is on the borderline of chilling and forcing. This relatively low temperature was used due to the fact that two older growth chambers that were originally set at higher temperatures failed while the experiment was already ongoing. The humidity was kept at 50% and the plants were watered every other day. The temperature underneath the plastic was checked several times during the experiment and was not found to be considerably higher than 10°C. The dates when first bud burst occurred, and when 50% and 100% of all buds had burst on a particular tree were recorded and observations were made daily, for a period of 123 days. Bud burst was defined as the first appearance of green leaf tips from between the opening bud scales (defined as stage 3 in Murray et al. 1989, Figure 2.3 D).



Figure 2.3 Stages of bud burst as observed in *Populus tremula* in Experiment 1. A: dormant bud, B-C: swelling and greening of the bud scales, D: green leaf tissue visible from between the scales (bud burst), E: leaf unfolding, F: juvenile leaves. Scale bar A-D: 2mm, E: 5mm, F: 8mm.

The second phase of the experiment began on 18th January 2010. The 30 plants used in the second experimental phase had received their full chilling requirement in natural conditions at the gardens of Teagasc Horticultural Centre (Kinsealy) and were placed in the Conviron PGR15 growth chamber. The same number of replicates (i.e. 5 trees per condition) and the same photoperiod, forcing temperature, humidity and watering conditions were used as in the first phase of the experiment. Observations were made for 68 days.

EXPERIMENT 2

As in the previous year, an experiment investigating dormancy release was conducted in the autumn and winter of 2010-2011. 48 3-4 year old aspen trees and 48 6-7 year old birch trees were used to determine the effects of photoperiod and chilling requirement on the timing and percentage of budburst. Experiment 2 involved three phases, consistent with three chilling duration conditions. See Table 2.1 for an overview of the different treatments in the two experiments of this study.

Table 2.1 Details of the treatments received in each of the experiments performed during two seasons. "Natural" refers to non-controlled temperatures and photoperiods, received outdoors. "Chilling duration" is expressed in days with average daily temperature $\leq 10^{\circ}\text{C}$, starting from 1st September.

Experiment	Plants		Chilling treatment		Forcing treatment		Recordings	
	Species	Number per treatment	Temperature and photoperiod	Duration (days)	Temperature ($^{\circ}\text{C}$)	Photoperiod (hours)	Frequency	Percentage bud burst
1	<i>P. tremula</i>	5	Natural	35, 82	10	8, 12, 16	Daily	1st, 50% and 100%
	<i>B. pubescens</i>							
2	<i>P. tremula</i>	8	Natural	25, 53, 76	20	10, 16	Every 2nd day	Exact count
	<i>B. pubescens</i>							

On the 4th November 2010, the first batch of 16 aspen and 16 birch trees were transferred from outdoor conditions to two controlled environment chambers in Trinity College Dublin (Conviron PGR15 and Conviron A1000), wherein two different photoperiod conditions were automatically regulated (10 and 16 hours of day light, with 8 trees of each species in each treatment). The temperature was kept at a constant 20 $^{\circ}\text{C}$ and the humidity at 50%. Observations of the exact number of buds that had burst on a particular tree were recorded every other day, for 101 days. During the months of September and October 2010 the plants had received 25 chilling days, before being transferred into experimental conditions (Table 2.1). One of the growth chambers had a thermostat failure in the beginning of December 2010, which made the plants in this chamber receive a heat shock treatment of up to 40 $^{\circ}\text{C}$ for several hours. Bud burst was recorded for these plants, and the results of this treatment (10

hour photoperiod treatment in birch) will be discussed and compared but cannot be analysed together with results of other 'regular' treatments.

For the second phase, 32 trees were brought from ambient into controlled conditions (two Conviron A1000 growth chambers) on the 12th December 2010. At this date, the trees had undergone 53 chilling days (Table 2.1). 16 trees from each species were used, of which eight trees received a photoperiod of 10 hours and eight plants were given 16 hours of daylight. Photoperiod, forcing temperature, humidity, watering treatment and observation taking were the same as in the first phase. The plants were monitored for 63 days as previously described.

The last 32 trees had theoretically fulfilled their chilling requirements with 76 days of chilling (Table 2.1, Figure 2.4), and were transferred into experimental conditions in the Conviron A1000 and PGR15 growth chamber on the 14th January. Observations were recorded for 30 days, and photoperiod, forcing temperature, humidity and watering regime were the same as previously described.



Figure 2.4 Dormant *P. tremula* trees kept in ambient conditions in December 2010, before transfer into controlled conditions.

2.2.3 Chilling unit calculations

The number of chilling days is a crude method for determining the amount of chilling that plants have received. A more accurate description is given by chilling units, which is used in this analysis. Daily chilling units were calculated using the chilling equation of the Dormphot

model (Caffarra et al. 2011b) for *B. pubescens*. The chilling state $CS(t)$ at day t , is given by the daily accumulation of chilling units:

$$CS(t) = \sum_{t_d}^t \frac{1}{1 + e^{aC(T_t - cC)^2 + (T_t - cC)}}$$

where t_d is the starting day of chilling accumulation (set as 1st September of the year preceding bud burst), T_t is the average daily temperature on day t , and aC and cC are function parameters, estimated as respectively 0.03 and 13.89 for *B. pubescens*. The critical threshold (C_{crit} , or fulfilment of chilling requirements) for *B. pubescens* was determined at $CS(t) = 55.35$ (Caffarra et al. 2011b).

For *P. tremula*, no species-specific models for chilling requirement are available; therefore a general method for calculation of chilling units for temperate trees was used. Chilling units were estimated as 1 if the average daily temperature was between 0 and 12°C, and estimated as 0 if temperatures were negative or above 12°C, starting from the first of September (e.g. Myking and Heide 1995, Battey 2000).

2.2.4 Meteorological data

Daily maximum and minimum temperatures of the outdoor chilling conditions were recorded at the Met Eireann meteorological station at Dublin Airport (53°25'N, 06°16'W, 74m a.s.l., 4.2km from Kinsealy Research Centre).

2.2.5 Thermal time calculations

An important difference between Experiment 1 and Experiment 2 was the forcing temperature used (Table 2.1): 10°C and 20°C respectively. Both experiments can therefore only be compared if the heat units received by the plants are standardised. This can be done by calculating the thermal time (TT) for each data point recorded for bud burst, and by using this measure in analysis instead of the number of days to bud burst. The thermal time to bud burst was calculated as the accumulated degree days above 0°C since transfer into experimental

conditions, or since 1st January for plants that were transferred after this date. Outdoor temperatures did not rise above any of the forcing temperatures before transfer.

The thermal time to bud burst was calculated for each bud burst recorded. Buds that did not flush during the observation period were recorded as flushing after this period for the purpose of statistical analysis (123 and 68 days for Experiment 1 and 101, 63 and 30 days for Experiment 2). The TT to first bud burst, 50% bud burst and 100% bud burst was subjected to a univariate 2-way ANOVA, with chilling, photoperiod, and their interaction as fixed effects, according to the linear model:

$$TT_{ij} = m + P_i + C_j + (P \times C)_{ij} + e_{ij}$$

where TT_{ij} is the thermal time to first bud burst, 50% bud burst or 100% bud burst under photoperiod i of trees with chilling units accumulation of j , m is the mean of TT_{ij} , P_i is the fixed effect of photoperiod i ($i = 8, 10, 12$ or 16 hours), C_j is the fixed effect of chilling units j until transfer into experimental conditions ($j = 46, 71, 84, 117$ or 125 for *B. pubescens* and $33, 60, 61, 84$ or 103 for *P. tremula.*, see Table 2.3), $P \times C$ is the fixed effect of the interaction between temperature and chilling duration, and e_{ij} is the random residual. The independence of observations (see 2.2.5) is not violated in this test, as the stages 1st bud burst, 50% bud burst and 100% bud burst was only measured once in each tree.

2.2.6 Statistical analyses

The timing (number of days starting from transfer into forcing conditions) and percentage of bud burst were measured in this study. In Experiment 1 percentage of bud burst was monitored daily and timing was noted down at three different stages: (1) moment of first bud burst on the tree, (2) bursting of 50% of the total number of buds on the tree, and (3) bursting of 100% of the total number of buds on the tree. In Experiment 2 the percentage of bud burst was recorded as an exact number of buds that had flushed against the total number of buds on the tree, and observations were made approximately every other day. The two response variables, days to bud burst (*BBD*) and percentage of bud burst (*percentage*), were analysed

in relation to two explanatory variables: the environmental factors (1) duration of chilling in natural conditions (*chilling*), and (2) photoperiod length during forcing (*photoperiod*). Other response variables used were *TT*, the thermal time to bud burst, and *slope*, the slope of a regression line between observation points (number of days to stage of bud burst vs. stage of bud burst: 1st bud burst, 50% bud burst and 100% bud burst).

The test statistics used were variations of a standard ANOVA (Analysis of Variance), which compares group means and their variances to test if group responses are significantly different and whether the differences are related to the explanatory variables. One of the conditions for standard ANOVA is that observations are independent (see section 2.2.5), while bud burst is intrinsically not independent within a single tree: a particular bud is more likely to open when other buds on the same tree have already flushed, since dormancy release has set in. Both experiments in this study involved repeated measures within-subjects (bud burst in the same individual trees) as well as a between-subjects design (created by the independent variables *chilling* and *photoperiod*), variations on standard ANOVA were used: (1) repeated-measures ANOVA (Tabachnick and Fidell 2007) and (2) mixed between-within subjects ANOVA (also known as a split-plot ANOVA) were carried out.

The concept of repeated-measures ANOVA is similar to a standard ANOVA, with the main difference that the two sources of variance are separated out: the variance within and the variance between subjects are both taken into account. The mixed ANOVA design however, is a combination of between-groups (i.e. standard) ANOVA and repeated-measures ANOVA. This design is used when the repeated measure is carried out on different levels (Pallant 2007), for instance as in the levels of bud burst in Experiment 1 (1st bud burst, 50% bud burst and 100% bud burst). For tests where the repeated measures were irrelevant, for instance when *slope* was used as response variable, a standard factorial or ANOVA was performed. All statistical analyses were carried out using SPSS 18.0 and R 2.12.2. Graphs were constructed using R.2.12.2 and SigmaPlot 12.0.

2.2.7 Assumptions for ANOVA

The general assumptions underlying ANOVA hypothesis testing require that: (1) the observations are taken from normally distributed populations, (2) the observations are taken from populations with homogeneity of variances, and (3) the observations are independent of one another. Besides these general assumptions, also the (4) homogeneity of inter-correlations among the levels of within-subjects needs to be tested for the mixed between-within subjects ANOVA, or the (5) homogeneity of within-subject variances for the repeated-measures ANOVA (sphericity).

1. The normality of the data was tested using Q-Q plots, where the expected normal values were plotted against the observed values.

2. Levene's tests were conducted in order to test the null hypothesis that the error variance of the dependent variable was equal across groups (homogeneity of variance). In some of the tests there was a violation of this hypothesis. However, ANOVA is robust to violations of this assumption, provided the size of the groups are similar (Stevens 1996), which is the case in these experiments, unless mentioned otherwise.

3. Independence of the samples was ensured by randomisation in the choice of plants used for each treatment. As mentioned before, observations of bud burst were not independent since days to bud burst was recorded several times within a single tree, in which case a repeated-measures ANOVA was carried out, or a mixed between-within subjects ANOVA.

4. In the mixed between-within subjects ANOVA, the homogeneity of inter-correlations among the levels of within-subjects was assessed using Box's M Test, with the null hypothesis that the observed covariance matrices of the dependent variables are equal across groups. In some cases there is a violation of this test (significance <0.05), however ANOVA is robust to violations of this assumption (which is sensitive to a slight non-normality), provided the size of the groups is similar (Tabachnick and Fidell 2007).

5. In repeated-measures ANOVA, the homogeneity of variance within the subjects was tested using Mauchly's test of sphericity. When sphericity could not be assumed (significance >0.05), a Greenhouse-Geisser correction of the degrees of freedom was used to calculate a meaningful significance for the F -value of the ANOVA (Pallant 2007).

2.3 RESULTS

2.3.1 Meteorological parameters

Monthly mean temperatures (Table 2.2 and Figure 2.5 A) and the accumulation of chilling units (Figure 2.5 B-C) during the experiments differed between the two seasons of the study (Experiment 1 and Experiment 2). In September and October, average monthly temperatures were higher during the chilling period of Experiment 2 than in Experiment 1, while the average November and December temperatures were lower in Experiment 2, compared to Experiment 1 (Figure 2.5 A, Table 2.2). Because temperatures close to the freezing point contribute less to the accumulation of chilling units, chilling was more advanced during the first year of the experiments for *B. pubescens* (Figure 2.5 B). For *P. tremula* a more crude method was used for the calculation of chilling units and the difference in chilling accumulation between both experiments was smaller than for *B. pubescens* (Figure 2.5 B and C). This is important for comparisons between both experiments: Not only forcing temperatures were different, but also chilling temperatures.

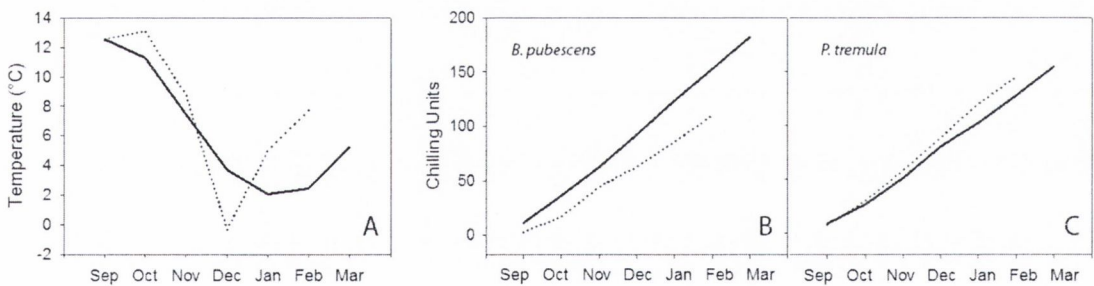


Figure 2.5 Mean monthly temperature at Dublin Airport (A) and accumulated chilling units (averaged per month) for *B. pubescens* (B) and *P. tremula* (C) during autumn and winter of 2009-2010 (Experiment 1, black line) and 2010-2011 (Experiment 2, dotted line).

Table 2.2 Mean monthly temperatures recorded at Dublin Airport during autumn and winter months of the experiments. The January temperature represents the mean temperature until the last day plants were taken into experimental conditions.

Experiment	Mean monthly temperatures (°C)				
	Sep	Oct	Nov	Dec	Jan*
1	12.6	10.1	7.4	3.7	1.3
2	13.2	11.3	5.3	0.3	4.3

*Until last date before transfer into experimental conditions

2.3.2 Data exploration

Before conducting a detailed statistical analysis of the data, an overview of the experimental results is presented in this section in the form of graphs and descriptive statistics (means and standard deviations) to become familiarised with the data and the variation found within the data. Certain trends can be discovered using these basic methods and visual tools for data exploration. These trends are described in this section and their significances are tested further on in sections 2.3.3 - 2.3.6. The variable *chilling* was expressed in chilling units, calculated for each species separately using the formulas described in section 2.2. The chilling units received by the plants per treatment and per species are reported in Table 2.3.

Table 2.3 Chilling units at the start date of transfer to experimental conditions for *B. pubescens* and *P. tremula* clones over the 2 experiments

Experiment 1			Experiment 2		
Date	Chilling units		Date	Chilling units	
	<i>B. pubescens</i>	<i>P. tremula</i>		<i>B. pubescens</i>	<i>P. tremula</i>
25/11/2009	71	60	04/11/2010	46	33
18/01/2010	125	103	12/12/2010	84	61
			14/01/2011	117	84

EXPERIMENT 1

In Figure 2.6, the response variable on the Y-axis *BBD* (days to bud burst) refers to the timing of bud burst of all recorded data points. Figure 2.6 A and 2.6 B show a clear difference in timing between the two chilling conditions in both species: bud burst was earlier in fully chilled trees (white boxes) than in partially chilled trees (grey boxes), and there were no overlapping values of *BBD*, except one outlier in the shortest chilling and photoperiod condition in *P. tremula* (Figure 2.6 B). From the size of the boxes, it is also apparent in Figure 2.6 that there was greater variation in the response variable *BBD* for the partially chilled trees compared to the fully chilled trees, in both species (Figure 2.6 A and 2.6 B). There was not a great variation in response between photoperiods. However, the number of days to bud burst was lower for the plants under long photoperiod after partial chilling in *B. pubescens* (Figure 2.6 A), which

might affect the significance of the influence of photoperiod on the timing of bud burst or the significance of an interaction between variables *chilling* and *photoperiod*. Whether there is a significant effect is tested further on in this chapter. A few outlying values of bud burst timing were recorded, indicated by the dots outside the boxes. For *P. tremula*, a high variation was found in the shortest chilling condition (Figure 2.6B, grey boxes), yet not many data points were recorded, since the frequency of bud burst within the monitoring time was extremely low (see section 2.3.3.3 on percentage of bud burst). In 12hrs of daylight for instance, only 2 buds flushed across the five trees in this condition.

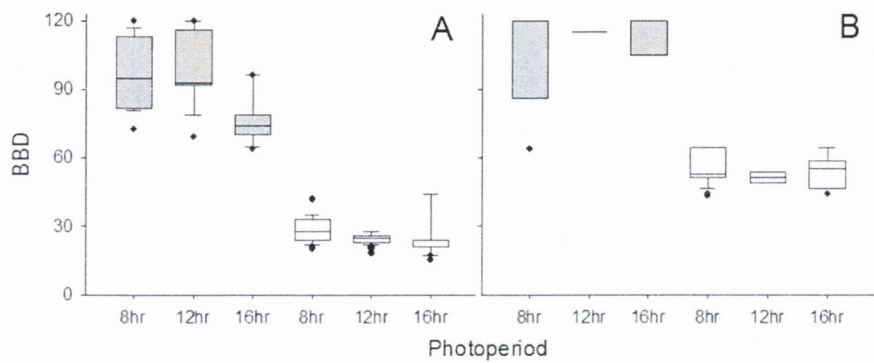


Figure 2.6 Box plot of days to bud burst (BBD) for different photoperiod conditions of *B. pubescens* (A) and *P. tremula* (B) in Experiment 1 (10°C forcing temperature), grouped by chilling condition (grey boxes: partially chilled, white boxes: fully chilled). Horizontal bars within the boxes represent the median. Outliers (dots) are shown in the 95th/5th percentile.

The comparison of the means and standard deviations of both species in their timing of bud burst after transfer into growth chambers (Figure 2.7), showed that birch had lower chilling requirements than aspen: after receiving the same chilling temperatures and duration (black dots for partial chilling and white dots for full chilling), the average values for bud burst were lower for birch (Figure 2.7 A) than for aspen (Figure 2.7 B), suggesting bud burst is earlier. Whether this difference is significant and which environmental factors are at the basis of this difference, is tested in section 2.3.4.

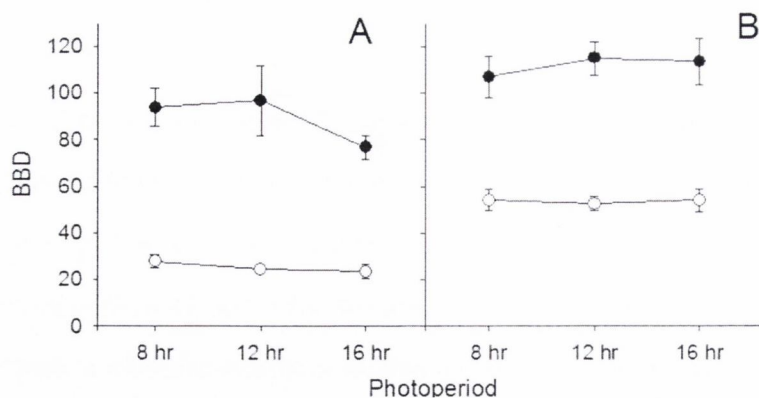


Figure 2.7 Days to bud burst (BBD) of *B. pubescens* (A) and *P. tremula* (B), under a range of photoperiod and chilling conditions (black dots: partially chilled, white dots: fully chilled). Dots are mean values and error bars represent the standard deviation.

The percentage of bud burst at the end of the experiment varied quite substantially between the two species (Figure 2.8). Nearly all birch trees reached 100% bud burst at the end of the monitoring period, while aspen trees had a much slower response. None of the aspen trees reached the stage of 50% bud burst within the monitoring period for the partially chilled conditions (black bars in Figure 2.8, values between 0 and 13%), while fully chilled aspens did show a greater bud burst response of between 60 and 85% (Figure 2.8, grey bars). The latter condition shows a large variation around the mean (large error bars), which is due to a substantial number of trees not flushing at all under the forcing temperature of 10°C, despite a long chilling period.

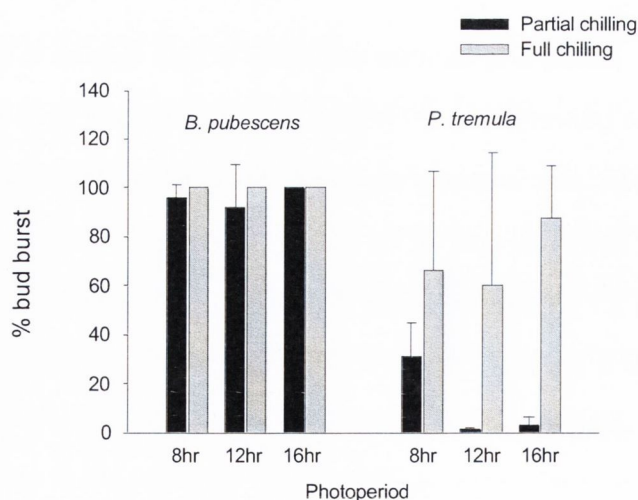


Figure 2.8 Mean percentage of bud burst at the end of Experiment 1 for *B. pubescens* and *P. tremula* clones in three photoperiod conditions (8hr, 12hr, and 16hr daylight) and two chilling conditions (black bars: partial chilling, grey bars: full chilling) Error bars represent standard deviation.

EXPERIMENT 2

Figure 2.9 presents all bud burst times (BBD) recorded for *B. pubescens* (Figure 2.9 A) and *P. tremula* (Figure 2.9 B) in Experiment 2. From this figure, it is clear that bud burst occurred earlier with increased chilling duration for both species: buds that received the least amount of chilling (data in grey boxes in Figure 2.9) flushed later than buds with intermediate chilling (white boxes) and much later than buds with most chilling (patterned boxes). Also the length of the photoperiod received during forcing conditions had a possible effect on the timing of bud burst, with earlier bud burst for buds under a longer photoperiod, although this trend is not present in the condition with longest chilling duration (patterned boxes in Figure 2.9), or for trees with the shortest chilling condition in *B. pubescens*: here, bud burst occurred earlier under shorter photoperiod (10hr) than under longer photoperiod (16hr). The trees in this treatment (10 hours of daylight and minimal chilling duration) received a heat shock due to mechanical failure. No visible trends can be noticed in the variation (box sizes) within each of the conditions (Figure 2.9 A and 2.9 B).

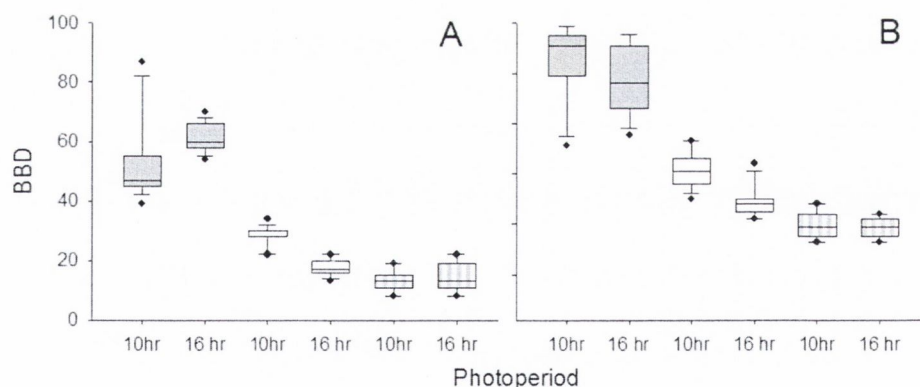


Figure 2.9 Box plot of days to bud burst (BBD) for different photoperiod conditions of *B. pubescens* (A) and *P. tremula* (B) in Experiment 2 (20°C forcing temperature), grouped by chilling duration (grey boxes: minimal chilling, white boxes: intermediate chilling, patterned boxes: full chilling). Horizontal bars within the boxes represent the median. Outliers (dots) are shown in the 95th/5th percentile. Far left box in A: heat shock treatment.

A comparison is made between both species in Experiment 2 in Figure 2.10. The treatment of *B. pubescens* plants with an accidental heat shock (46 chilling units and 10 hours of daylight) is not depicted in the figure. The average number of days to bud burst was 52 days for this condition, with a standard deviation of 13. More variation in days to bud burst was observed in

P. tremula (Figure 2.10 A) than in *B. pubescens* (Figure 2.10 B), indicated by the larger error bars for *P. tremula*. The mean bud burst date was generally slightly later in *P. tremula* compared to *B. pubescens*, except for the longest chilling duration, where the difference in mean days to bud burst was not very clear.

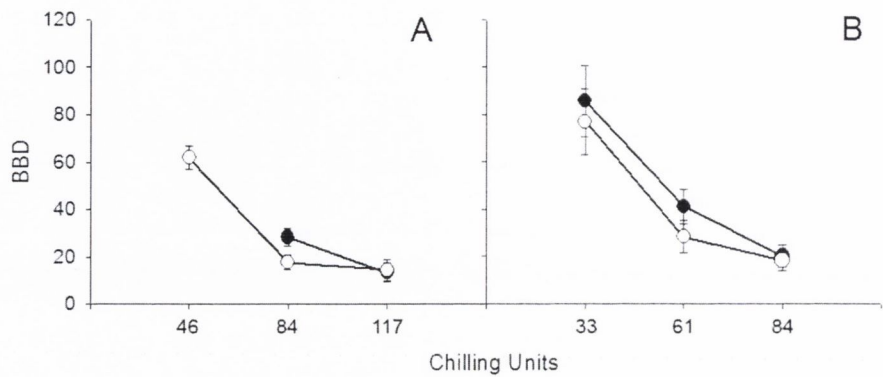


Figure 2.10 Days to bud burst (BBD) of *B. pubescens* (A) and *P. tremula* (B), under a range of chilling units received before transfer into forcing conditions and photoperiod conditions (black dots: 10 hours of daylight, white dots: 16 hours of daylight). Dots are mean values and error bars represent the standard deviation.

The percentage of bud burst at the end of the monitoring period (after 103 days for minimal chilling condition, 63 days for intermediate chilling and 31 days for maximal chilling) is illustrated in Figure 2.11. A great difference can be detected from this figure between percentage of bud burst in the minimal chilling condition and intermediate and maximal chilling. Percentage of bud burst is noticeably lower compared to the other two conditions, except for the short photoperiod condition (10 hours of daylight) in *B. pubescens*, which is the condition with heat shock treatment. Because the monitoring period was unusually long compared to other publications reporting on similar experiments (e.g. Heide 1993, Myking and Heide 1995, Ghelardini et al. 2009, Caffarra and Donnelly 2010), a cut-off time was decided on 60 days in further analyses comparing bud burst percentages in the different treatments.

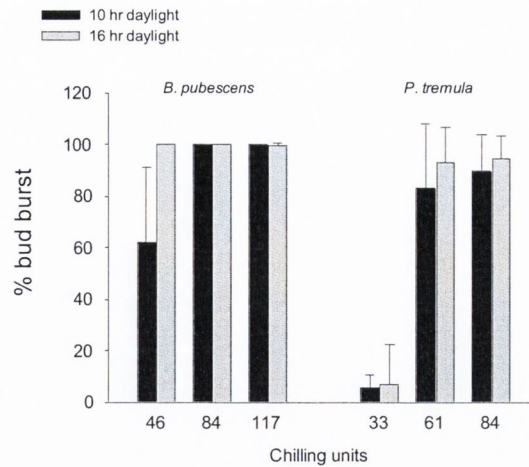


Figure 2.11 Mean percentage of bud burst at the end of Experiment 2 for *P. tremula* and *B. pubescens* clones in three chilling conditions, expressed in chilling units and two photoperiod conditions (black bars: 10hr, grey bars: 16hr daylight) Error bars represent standard deviation.

2.3.3 Speed of bud burst

2.3.3.1 Influence of chilling and photoperiod on the speed of bud burst

The speed at which bud burst occurred was determined by evaluating the slopes of a linear regression line through the data points of each percentage of bud burst measured. In order to compare the data from Experiment 1 and Experiment 2, only the points of first bud burst, 50% bud burst and 100% bud burst were analysed, even though continuous bud burst percentage values were recorded during Experiment 2. The analysis of continuous bud burst percentage is described further in this section. Here, four separate univariate two-way ANOVAs were performed with the slope of the linear regression line as the response variable and with *chilling* (two chilling durations for *B. pubescens* in Experiment 1; and three chilling durations for *P. tremula* and *B. pubescens* in Experiment 2) and *photoperiod* (three conditions in Experiment 1 and two conditions in Experiment 2) as explanatory variables (Table 2.5). Heat shock birches (Experiment 2) were omitted from the analyses, as was the condition with the shortest chilling duration in Experiment 1 for *P. tremula*. Slopes could not be calculated for this condition, as most trees did not reach 50% bud burst within the recording period. The data for first bud burst were analysed further. All tests for homogeneity of variances (Levene's test) were non-significant ($p > 0.05$), except for *P. tremula* in Experiment 2 ($p: 0.01$), however

group sizes were equal under this condition and therefore homogeneity of variances could be assumed.

Table 2.5 Average values ($\pm$ standard deviation) for the speed of bud burst (the slope of a linear regression line between first bud burst, 50% bud burst and 100% bud burst) for conditions with different chilling durations (CD, chilling days) and photoperiod lengths (Photo); and Analysis of Variance results for the effects of photoperiod (P), chilling duration (C) and interaction between both effects (C $\times$ P) on the speed of bud burst. Bold *p*-values are significant.

Average values				ANOVA results				
Photo (hr)	CD (days)	<i>B. pubescens</i>	<i>P. tremula</i>	Effect	<i>B. pubescens</i>		<i>P. tremula</i>	
					<i>F</i> (<i>df</i>)	<i>p</i>	<i>F</i> (<i>df</i>)	<i>p</i>
Experiment 1								
8	35	15.0 ± 6.4	/	C	55.21 (1)	<0.001	/	/
8	82	3.9 ± 2.5	8.6 ± 2.6					
12	35	18.3 ± 2.7	/	P	2.94 (2)	0.073	0.67 (2)	0.537
12	82	2.8 ± 0.8	7.3 ± 2.9					
16	35	8.6 ± 4.3	/	CxP	5.48 (2)	0.011	/	/
16	82	4.4 ± 3.2	9.6 ± 2.3					
Experiment 2								
10	25	/	42.9 ± 19.2	C	2.96 (2)	0.067	22.10 (2)	<0.001
10	53	4.6 ± 1.4	22.4 ± 19.6					
10	76	6.5 ± 1.2	6.3 ± 2.2	P	2.95 (1)	0.096	3.64 (1)	0.064
16	25	6.5 ± 3.1	32.0 ± 16.0					
16	53	4.6 ± 2.7	11.1 ± 6.7	CxP	2.95 (1)	0.096	0.94 (2)	0.401
16	76	3.8 ± 1.1	6.3 ± 2.2					

The analyses of variance revealed significant main effects for chilling duration on the speed of bud burst for *B. pubescens* in Experiment 1, and for *P. tremula* in Experiment 2 (*p* = 0.000). For *B. pubescens* the significance level for *chilling* was close to 0.05 (*p* = 0.067), however excluding one condition might have influenced the results of the analysis. No significant main effects on speed of bud burst were discovered for photoperiod in any of the species or experiments. However, long photoperiods tended to reduce the number of days to bud burst although this trend was only statistically significant (*p* = 0.073, 0.096 and 0.064) at the 0.10 level. An interaction between *chilling* and *photoperiod* had a significant effect on the speed of bud burst (*p* = 0.011) in *B. pubescens*, showing that the effect of chilling duration varied with different photoperiods in Experiment 1 (see Figure 2.7 A). Since the percentage of bud burst does not progress in a linear manner (see further), but rather forms a sigmoid response curve, a different analysis was performed to confirm the effects of *chilling* and *photoperiod* on the speed of bud burst, a repeated-measures ANOVA. In contrast, no significant interaction between *photoperiod* and *chilling* was found in Experiment 2 for *B. pubescens*. This difference

between the two experiments might be attributed to the higher forcing temperature used in Experiment 2, which could have masked the effect of *photoperiod*.

2.3.3.2 Influence of chilling and photoperiod on the difference in days to bud burst stages

The speed of bud burst was also measured as the number of days to three different stages of bud burst within a single tree: first bud burst, 50% bud burst and 100% bud burst. Smaller differences between the stages are measured when bud burst is faster (fast succession in days), compared to higher differences when bud burst is slower (more days needed to reach the next stage). Observations were made at several times on the same trees and are therefore not independent. Repeated-measures ANOVA was used to discover which of the explanatory variables (*chilling* or *photoperiod*) were responsible for the differences in days between the stages within a tree.

A repeated-measures ANOVA was performed for *B. pubescens* in Experiment 1 and 2 and for *P. tremula* in Experiment 2. Bud burst was rare in Experiment 1 for *P. tremula*, which resulted in many missing values for this condition and was therefore omitted from this analysis. The repeated within-subject factor (dependent variable) was the number of days after transfer into the growth chambers until each of the three stages of bud burst was reached (three levels of the dependent variable), and the factors were (1) *photoperiod* and (2) *chilling*. A summary of the degrees of freedom (*df*), F-values and *p*-values for the within-subjects factors is presented in Table 2.6. The assumption of sphericity was not met; therefore the *df* and *p*-values were adjusted according to Greenhouse and Geisser (1959, in: Pallant 2007).

Table 2.6 Repeated-measures ANOVA within-subjects results for the variable ‘numbers of days until first bud burst, 50% and 100% bud burst’ (with 3 levels) for *B. pubescens* and *P. tremula* clones under different chilling durations (C) and photoperiod conditions (P). Significant *p*-values (<0.001) are in bold. Degrees of freedom (*df*) and *p*-values were Greenhouse-Geisser adjusted to meet the requirements of sphericity.

ANOVA results				
Effect	<i>B. pubescens</i>		<i>P. tremula</i>	
	<i>F</i> (<i>df</i>)	<i>p</i>	<i>F</i> (<i>df</i>)	<i>p</i>
Experiment 1				
C	37.50 (1.74)	<0.001		
P	2.40 (3.48)	0.78		
CxP	4.70 (3.48)	<0.001		
Experiment 2				
C	2.37 (2.53)	0.095	42.63 (3.10)	<0.001
P	2.15(1.26)	0.146	2.51 (1.55)	0.102
CxP	1.45 (1.26)	0.242	0.49 (3.10)	0.697

Basically, the same conclusions can be made as from the analyses of the slopes of a linear regression line. The significant effects were (1) the main effect of *chilling* on the speed of bud burst of birch in Experiment 1 and of aspen in Experiment 2, and (2) the interaction of *chilling* and *photoperiod* on the speed of bud burst of birch in Experiment 1. It has to be kept in mind that these effects explain the variation within a single tree and not between trees in the same condition. A mixed within-between subjects ANOVA is needed to discover these effects (see further).

2.3.3.3 Percentage of bud burst

In Experiment 2, the exact percentage of bud burst was recorded approximately every two days, therefore it was possible to plot the percentage of bud burst against days in order to visualise the progression of bud burst. Previously, the slopes of linear regression lines between the days to three percentages of bud burst were calculated in order to analyse the differences in the speed of bud burst between the treatments. Here, it is shown that linear regression lines are only an approximation of the progression of bud burst. Percentage of bud burst was plotted against number of days to bud burst and the resulting trends exhibit a sigmoid rather than a linear pattern (Figure 2.12).

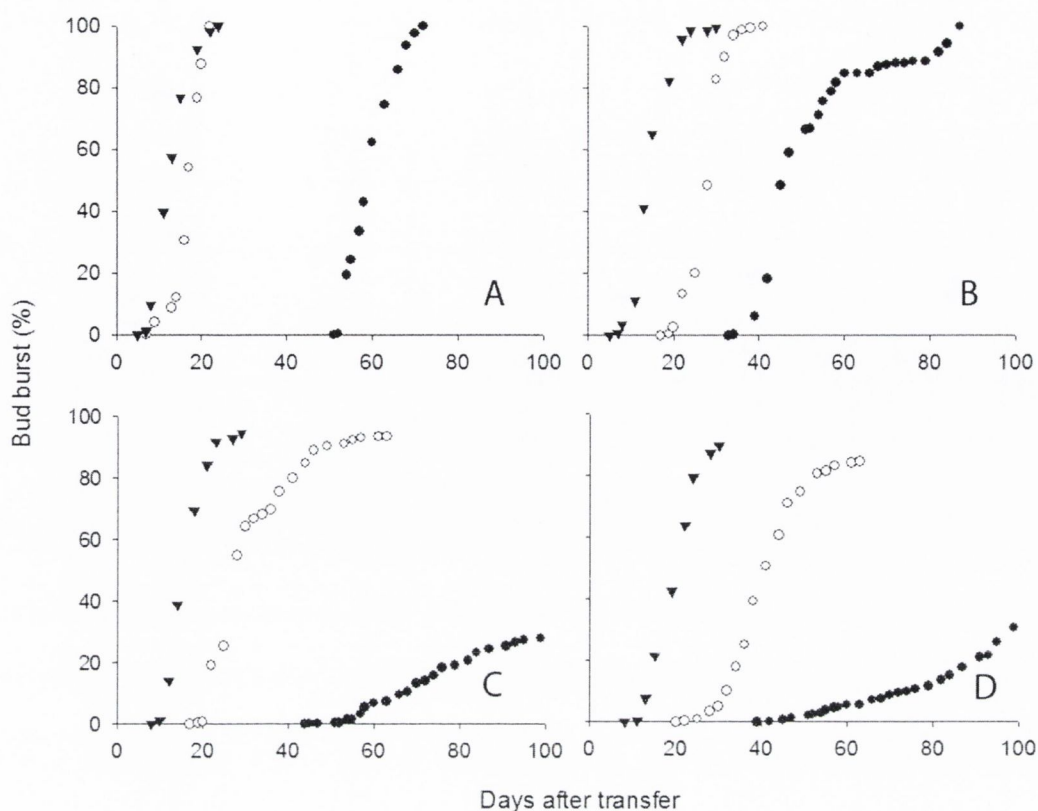


Figure 2.12 Percentage of bud burst in *B. pubescens* (A-B) and *P. tremula* (C-D) clones against number of days after transfer into experimental conditions in Experiment 2. A and C: 16 hr daylength, B and D: 10 hr daylength, *: minimal chilling, O: intermediate chilling, ▼: maximal chilling.

From these plots, it is also clear how chilling duration has an effect on the speed of bud burst in *P. tremula* (Figure 2.12 C-D). Particularly the shortest chilling condition had a much shallower slope compared to the other two chilling conditions, indicating a slower progression in bud burst. In *B. pubescens* on the other hand no significant effects were discovered for chilling on speed of bud burst (Tables 2.5 and 2.6), which is also shown in Figure 2.12 A-B: the sigmoid curves have a similar shape and steepness, except the minimal chilling condition in Figure 2.12 B. The lower part of this curve is steeper than the upper part, indicating an initial surge in bud burst, after which a slower progression of bud burst occurred. This aberrant behaviour is more than likely due to the heat shock that the trees in this condition received early on in the experiment.

2.3.4 Level of bud burst

Days to first bud burst, 50% bud burst and 100% bud burst were analysed using univariate two-way ANOVA with *chilling* and *photoperiod* as factors for each stage, species and experiment separately (Table 2.7). The equality of variances was tested using a Levene's test of equality, and confirmed for most ANOVAs. The equality of variances could not be assumed for *B. pubescens* in Experiment 2 (1st bud burst and 50% bud burst). Moreover, the groups were uneven in these tests, since the heat shock treatment was not part of the analysis. Therefore, all birches in this chilling condition were removed from the analysis, and degrees of freedom changed from 2 to 1 for chilling (C; Table 2.7). Significant *p*-values for the effect of all three factors (chilling, photoperiod and the interaction of chilling and photoperiod, see Table 2.7) on the timing of first bud burst were found for *B. pubescens* in Experiment 2. However, none of the three factors were significant for the date of first bud burst in this species in Experiment 1. Also for *P. tremula*, a significant influence of *chilling* was discovered on 1st bud burst date in Experiment 2, which was not present in Experiment 1 (Table 2.7). For days to 50% bud burst, a stage often recorded in phenological monitoring programmes, there was a significant effect of *photoperiod* for *B. pubescens* in Experiment 1 and for *P. tremula* in Experiment 2. Chilling duration had a significant influence on the timing of the stage of 50% bud burst for both species in Experiment 2. In all analyses, missing values (non-flushing buds) were considered as flushing at the latest date of the experimental recording time, except for *P. tremula* in Experiment 1, where bud flush was extremely rare after the shortest chilling duration. Therefore, the effect of chilling duration could not be measured for 50% and 100% bud burst in Experiment 1. In Experiment 2 however, the higher forcing temperature resulted in a higher percentage of bud burst, even after shorter chilling durations, and both *chilling* and *photoperiod* had significant effects on the timing of 50% bud burst and 100% bud burst for *P. tremula*. However, the condition with the shortest chilling duration in Experiment 2 had to be omitted from the 100% bud burst analysis for *P. tremula* due to the low number of plants reaching this stage, again resulting in 1 degree of freedom for the *chilling* factor. *B. pubescens* reached 100% bud burst at a timing that was influenced by *chilling* and *photoperiod* in both experiments and by interaction of both effects in Experiment 1.

Table 2.7 Univariate 2-way ANOVA results for the timing of three stages of bud burst (1st bud burst, 50% of buds burst and 100% of buds burst) in species *B. pubescens* and *P. tremula*, in two different experiments with varying chilling duration conditions (C; two conditions in Experiment 1 and three conditions in Experiment 2) and under different photoperiods (P; three conditions in Experiment 1 and two conditions in Experiment 2). Significant *p*-values are in bold (<0.05).

Effect	1st bud burst				50% bud burst				100% bud burst			
	<i>B. pubescens</i>		<i>P. tremula</i>		<i>B. pubescens</i>		<i>P. tremula</i>		<i>B. pubescens</i>		<i>P. tremula</i>	
	<i>F</i> (<i>df</i>)	<i>p</i>	<i>F</i> (<i>df</i>)	<i>p</i>	<i>F</i> (<i>df</i>)	<i>p</i>	<i>F</i> (<i>df</i>)	<i>p</i>	<i>F</i> (<i>df</i>)	<i>p</i>	<i>F</i> (<i>df</i>)	<i>p</i>
Experiment 1												
C	0.37 (1)	0.547	1.97 (1)	0.174	1.33 (1)	0.261	/	/	10.12 (1)	0.004	/	/
P	0.21 (2)	0.808	0.76 (2)	0.481	3.86 (2)	0.036	0.03 (2)	0.973	5.30 (2)	0.013	0.71 (2)	0.52
CxP	0.05 (2)	0.955	0.34 (2)	0.716	1.74 (2)	0.199	/	/	4.62 (2)	0.021	/	/
Experiment 2												
C	50.88 (1)	<0.001	86.6 (2)	<0.001	13.17 (1)	<0.001	211 (2)	<0.001	268 (2)	<0.001	7.83 (1)	0.009
P	13.83 (1)	0.001	0.03 (1)	0.855	0.02 (1)	0.901	6.05 (1)	0.018	18.45 (1)	<0.001	5.03 (1)	0.033
CxP	14.76 (1)	0.001	2.02 (1)	0.146	10.05 (1)	0.818	2.15 (1)	0.129	1.18 (1)	0.287	0.79 (1)	0.383

2.3.5 Effect of chilling on thermal time to bud burst

The effect of chilling duration on the timing of bud burst is illustrated in Figure 2.13. Bud burst is expressed in thermal time instead of days to bud burst, so that data of both experiments could be shown together, since different forcing temperatures were used in Experiment 1 (10°C) and Experiment 2 (20°C). A detailed and meaningful statistical test cannot be carried out on all these data together, because a different number of plants were used in each condition and a different number of chilling and photoperiod conditions was also used, which makes for an unbalanced design. However, using the crude method of comparing the mean values in Figure 2.13, it is clear that overall chilling duration had an effect on the timing of bud burst in the three stages (1) 1st bud burst, (2) 50% bud burst and (3) 100% bud burst. Thermal time to bud burst decreased after longer chilling periods for all stages, even though chilling was more frequently a significant factor on the timing of the different stages of bud burst in Experiment 2 than in Experiment 1 (Table 2.7).

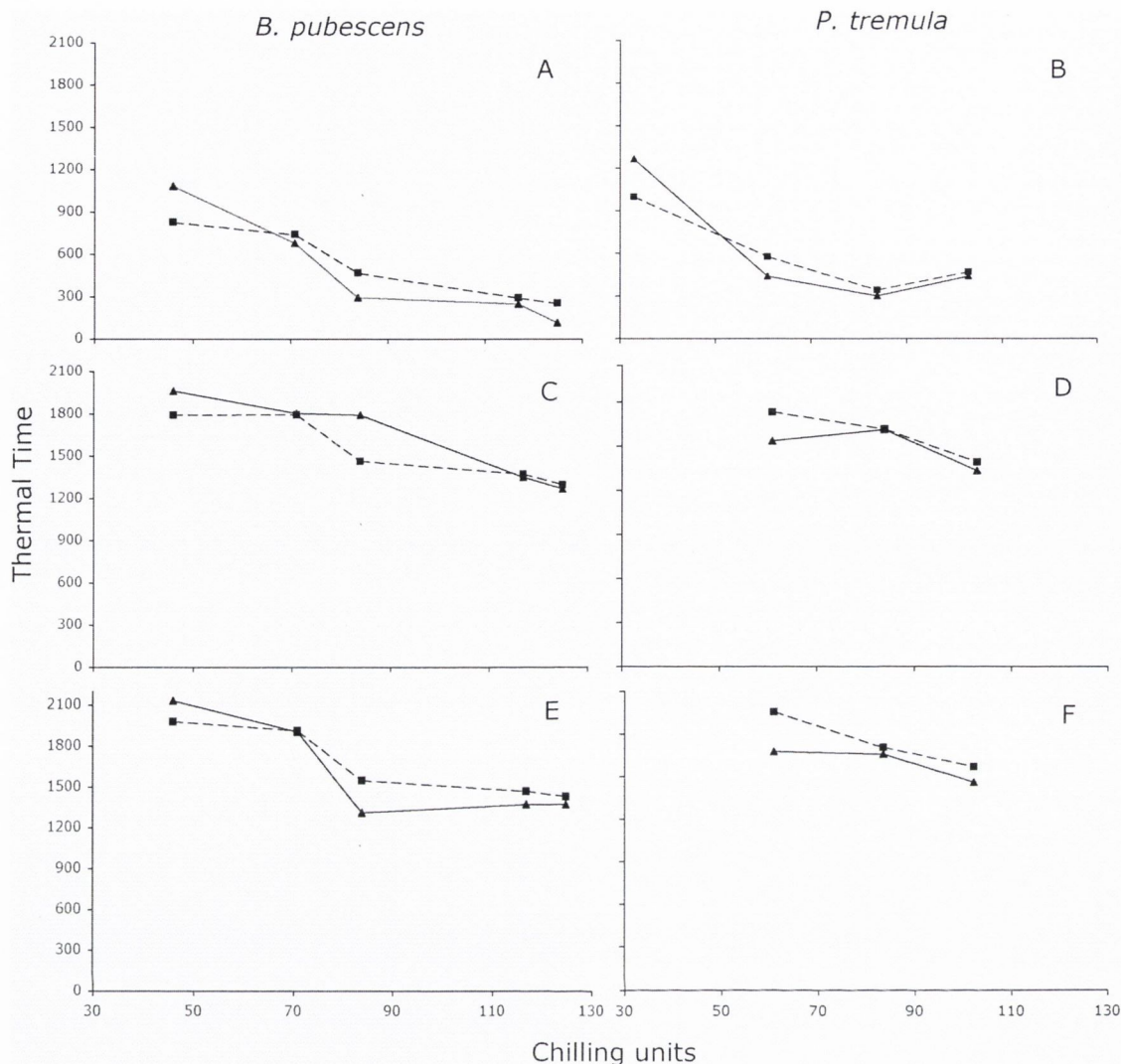


Figure 2.13 Average thermal time (expressed in degree-days $>0^{\circ}\text{C}$) to (A-B) first bud burst, (C-D) 50% bud burst and (E-F) 100% bud burst for *B. pubescens* (A, C and E) and *P. tremula* (B, D and F) clones subjected to a total of five chilling conditions for *B. pubescens* (46, 71, 84, 117 and 125 chilling units) and a total of four chilling conditions for *P. tremula* (33, 60-61, 84 and 103 chilling units). --■--: thermal time to bud burst averaged over conditions with 8-10 hr photoperiod, —▲—: thermal time to bud burst averaged over 12-16 hour photoperiod. Data are from both Experiment 1 and Experiment 2.

2.3.6 Overall effects of chilling and photoperiod on days to bud burst

For testing the between-subjects (between trees in these experiments) effects of *chilling* and *photoperiod* on the timing of bud burst, mixed between-within subjects ANOVA were performed for Experiment 1 (where days to bud burst was recorded only at 3 stages of bud burst) and Experiment 2 (with continuous measurements of days to bud burst) separately. The difference between both tests in this case is days to bud burst in Experiment 1 is regarded as a 'score' at a certain level (1st bud burst, 50% bud burst, 100% bud burst), and in

Experiment 2 as a continuous response. Earlier the within-subjects effect of chilling and photoperiod on the level of bud burst were determined, while here the interest lies in the effect between the trees (in Experiment 1) and between the buds, regardless of which tree the buds are on (in Experiment 2). An advantage is that using a mixed effect model missing values are not a problem, while individuals with missing data were removed from the within-subjects ANOVA.

EXPERIMENT 1

Bud burst only occurred sufficiently in *B. pubescens* to carry out this test. Because of the method of data collection, days to bud burst cannot be considered as a continuous response, but is rather considered in this test as a 'score' that was achieved at three stages: 1st bud burst, 50% bud burst and 100% bud burst. Therefore, the independent variables were (1) *chilling* (C), (2) *photoperiod* (P), but also (3) *level of bud burst* (BB). These variables were entered in the analysis as main fixed effects because the environmental factors and levels were 'imposed' on all individual trees in the same manner. The random effect was the individual subject, or *tree* in this experiment (considered as a random sample), and the response variable was the days to each level of bud burst. The question of interest was whether the individual trees showed a different change in budburst over the three stages for the different treatments. For the between-subjects multivariate test a Wilks' Lambda statistic was used, which showed there was a significant interaction between bud burst and chilling ($p < 0.05$, Table 2.9). The interaction between chilling and photoperiod was not significant. There was a substantial main effect for *level of budburst* and for *chilling* ($p < 0.001$), and also a significant influence of *photoperiod* ($p < 0.05$), indicating that the birch trees needed a significantly different number of days to reach each stage of bud burst, and this difference was due to the different chilling and photoperiod treatments. A significant interaction between *level of bud burst* and *chilling* was found ($p < 0.001$), and also an interaction between all three variables tested ($p < 0.05$), although the effect of *photoperiod* on days to each level of bud burst is minor, indicated by the non-significant p -value of interaction between *level of bud burst* and *photoperiod* ($p = 0.177$). This implies that the trees responded differently to the

chilling conditions for different levels of bud burst, for instance chilling had a more significant effect on the 100% bud burst level than on the other two levels of bud burst in *B. pubescens* (see Table 2.7).

Table 2.9 (A) Average values of number of days to stage of bud burst (BB) with standard deviation, for different chilling and photoperiod conditions of *B. pubescens* in Experiment 1; and (B) Multivariate mixed between-within subjects ANOVA results for the fixed factors effects on timing of bud burst. A Wilk's Lambda statistic was used for the Multivariate test. BB: bud burst stage, C: chilling condition, P: photoperiod. Significant *p*-levels are in bold.

A.						
	Partial chilling			Full chilling		
	Photoperiod					
	8hr	12hr	16hr	8hr	12hr	16hr
BB						
1st	77.4 ± 9.3	73.6 ± 10.9	63.0 ± 13.6	80.8 ± 2.8	78.2 ± 1.5	76.0 ± 3.5
50%	90.4 ± 16.5	92.6 ± 10.7	74.5 ± 9.1	84.2 ± 4.6	82.0 ± 1.2	79.4 ± 4.3
100%	97.0 ± 17.3	107 ± 11.6	80.3 ± 10.9	88.6 ± 7.8	83.8 ± 1.3	84.8 ± 9.7

B.			
Fixed effects	Wilks' Lambda		
	<i>d</i>	<i>F</i>	<i>p</i>
BB	2	85.58	0.000
C	1	329.13	0.000
P	2	3.71	0.043
BB x C	2	25.84	0.000
BB x P	4	1.67	0.177
C x P	2	2.35	0.121
BB x C x P	4	3.04	0.029

EXPERIMENT 2

All bud burst data from Experiment 2, excluding the heat shock treatment of *B. pubescens*, were used in a mixed between-within subjects ANOVA analysis. The days to bud burst data was continuous in this experiment, as opposed to Experiment 1 and therefore the only main fixed effects were *chilling* and *photoperiod* in this test. The dependent variable was *days to bud burst* and the subject variable was *tree*, in which each bud burst date represented a repeated measure. The random factor in this case was *bud*. The test statistic used was a type III sum of squares test, and the results of this test are presented in Table 2.10, along with the

average number of days to bud burst and standard deviation for each condition in Experiment 2. All effects tested, with the exception of the main effect of *photoperiod* for *B. pubescens*, but including all interaction effects were highly significant ($p<0.001$). These results imply that chilling duration had a significantly strong effect on the date of bud burst, across all buds and in both species tested. The effect of *chilling* on the day of bud burst was strongly dependent ($p<0.001$) on the photoperiod during forcing for both species. In Figure 2.10, it was already visible that for longer photoperiods, the effect of a longer chilling duration on bud burst is weaker, as shown by the non-parallel lines in the plot. For *P. tremula* the main effect of photoperiod length during forcing was also significant on flushing dates for all buds, regardless of which tree the buds belonged to. As mentioned, for *B. pubescens*, *photoperiod* had no significant effect, which might have been due to the exclusion of the trees that received the heat shock treatment.

Table 2.10 Experimental results (mean number of days to bud burst and standard deviations) and mixed between-within subjects ANOVA results of Type III test for the fixed effects on the timing of bud burst for *B. pubescens* and *P. tremula* clones in Experiment 2. P: photoperiod, MC: minimal chilling, IC: intermediate chilling, FC: full chilling, C: chilling duration. Significant *p*-values (<0.001) are in bold.

Number of days to bud burst							Mixed ANOVA results			
P:10hr			P:16hr			Fixed effects	Type III sum of squares			
MC	IC	FC	MC	IC	FC		df	F	p	
<i>B. pubescens</i>										
Mean	/	28	13	56	28	18	C	2	5051.5	<0.001
St. Dev.	/	3	3	4	6	4	P	1	0.81	0.37
							CxP	2	219.14	<0.001
<i>P. tremula</i>										
Mean	52	41	20	57	17	14	C	2	9009.3	<0.001
St. Dev.	5	6	4	2	2	4	P	1	305.09	<0.001
							CxP	2	134.64	<0.001

2.4 DISCUSSION

This study reports on the experimental effects of two environmental factors on the timing of dormancy release, in the form of bud burst, in two important forest species native to Ireland and to Europe: *B. pubescens* and *P. tremula*. Novel insights and questions have arisen from this study, which have potential implications for forest conservation and management practices, and for the commercial growth of these two species used as renewable resources, particularly in the face of present and future climatic changes.

2.4.1 *The effect of chilling duration on bud burst*

An overall decrease in time to bud burst with increasing duration of chilling was found in at different forcing temperatures (Experiment 1 and Experiment 2), and for both species *B. pubescens* and *P. tremula*. This confirms previously found results (e.g. Heide 1993, Murray et al. 1989) and is an indication that dormancy release, as dormancy itself, is not a state in which a plant can be at a certain time, but involves continuous physiological changes within the plant. The largest differences in bud burst timing occurred in the early stages of chilling, which supports the use of an inverse exponential function to explain the relationship between accumulated chilling units and heat unit requirements for bud burst (Murray et al. 1989, Chuine et al. 1999, Ghelardini 2009).

Chilling duration not only had a significant effect on the timing but also on the speed at which bud burst occurred. This effect was more pronounced for *P. tremula* than for *B. pubescens* in Experiment 2 (see Figure 2.12): the trees receiving the shortest chilling durations only advanced very slowly to a higher percentage of bud burst, and even failed to reach 40% bud burst within the monitoring time of 103 days. The implications of such an extended period of budburst, or the time between 1st bud burst and 100% bud burst, for incompletely chilled plants might be harmful for trees, and particularly for young trees such as the ones used in this experiment. For example, in a natural forest, they can be out-competed very easily by

older trees with a wider canopy, and thus with more opportunities to photosynthesise early on in the growing season.

In Norway spruce, chilling duration was shown to have no effect on the ability of the trees to release bud dormancy, although bud burst advanced with longer chilling durations (Søgaard et al. 2008). Also in this study, birch showed a high percentage of bud burst, even after short chilling periods, while many of the aspen trees failed to show signs of dormancy release after the same chilling time and temperatures. This suggests that *P. tremula* has a higher chilling requirement than *B. pubescens*. But also in fully chilled plants, a longer time was needed for bud burst to occur in *P. tremula*, compared to *B. pubescens*. This implies that *P. tremula* also has a greater thermal time requirement, or alternatively, it is possible that full chilling requirements were not met for *P. tremula* at the latest date of transfer into experimental conditions (18th January for Experiment 2), resulting in an incomplete release of dormancy. Further testing is needed to assess the real chilling requirement of *P. tremula*.

Therefore, this research has shown that for *B. pubescens* the minimal chilling requirement was met in early-December but that chilling may not have been completed even by late-January for *P. tremula*, since not all aspen trees that were chilled until the 18th January reached 100% bud burst. Therefore, chilling requirements can be estimated from these experiments as >103 chilling units for *P. tremula* and <71 chilling units for *B. pubescens*. Heide (1993) observed a similar phenomenon whereby bud burst for *B. pubescens* was earlier compared to *P. tremula* in natural outdoor conditions.

2.4.2 Interaction effects between chilling duration and photoperiod length

Overall, fully chilled plants were less sensitive to photoperiod variations, indicated by the many significant values found for interaction effects between chilling duration and photoperiod length, and by the non-parallel lines in plots 2.7 and 2.10, which are quintessentially interaction plots. In Experiment 2 (20°C forcing temperature), some significant interaction effects were found for both species examined. In contrast, Heide (1993b) did not report any

interaction effects between chilling duration and photoperiod in a range of forest species, including *B. pubescens* and *P. tremula*. However, Heide (1993b) used cuttings carrying only a few buds in his experiments, as opposed to whole juvenile trees in these experiments.

2.4.3 The effect of photoperiod on bud burst

Because temperature is a variable measurement, it is a very unreliable indicator for favourable growing conditions for plants, particularly in temperate regions where a late frost can have a detrimental effect on the vulnerable tissues of newly opened leaf and flower buds. Also in subsequent seasons after frost damage, a delay in bud break, reduction in shoot and canopy growth, and a reduction in flowering and fruit production have been reported (Bokhorst et al. 2009). Therefore, plants in these regions use an extra security factor against the breaking of dormancy happening too early in the season: photoperiodism (Körner 2006, Körner and Basler 2010). However, to what extent a particular species is controlled by photoperiod can vary: for instance, some species with little or no day length control are *Sorbus aucuparia* L., *Rubus* spp. and *Carpinus betulus* L. (Heide 1993 a,b). Also for elm (*Ulmus* spp.), photoperiod had no effect on dormancy release (Ghelardini et al. 2009). However, for *F. sylvatica* photoperiod seemed to act as a limiting factor during the winter months, preventing early dormancy release and frost risk (Caffarra and Donnelly 2010), and also *Prunus* species exhibit a photoperiodic response in leaf formation (Heide 2008).

Overall, few significant effects of photoperiod length on the timing of bud burst were found between the two species studied here and no effect of photoperiod was found on the speed of bud burst: the advancement of bud burst within a single tree was not different for longer photoperiods compared to shorter photoperiods. In Experiment 1 (with low forcing temperature), the birch trees responded significantly different to different photoperiods in days to bud burst, while in Experiment 2 (high forcing temperature) only the aspen trees showed an effect of photoperiod. A photoperiod sensitivity of birch trees, particularly in the early stages of the chilling period, was reported in earlier studies (Håbjørg 1972, Li et al. 2003, Caffarra and Donnelly 2010), and therefore it is surprising that Experiment 2 did not provide evidence for a

significant effect of photoperiod on bud burst in *B. pubescens*. Results for significant effects of photoperiod might have been obscured due to the fact that all conditions for a successful release of dormancy were already met early on in the experiment for *B. pubescens* in Experiment 2 and any differentiation due to photoperiod is overridden; while in experiment 1, many *P. tremula* trees failed to break out of dormancy altogether. Several causes for these observations are possible: (a) as mentioned before, *P. tremula* might have higher chilling requirements than *B. pubescens* and than were met in Experiment 1, (b) *P. tremula* might have higher thermal time requirements than *B. pubescens* and than were provided with a constant temperature of only 10°C in Experiment 1, (c) the forcing temperature of 20°C in Experiment 2 was sufficient for *B. pubescens* for complete dormancy release even after insufficient chilling (d) *B. pubescens* is faster in the accumulation of chilling units and/or heat units than *P. tremula*, even when subjected to the same temperatures. This last explanation is a possibility, even though in Figure 2.5 the slopes of chilling unit accumulation lines do not differ greatly between the two species, the estimation for *P. tremula* was made using a very general function.

Besides chilling and/or thermal requirements, another explanation for *B. pubescens* failing to show sensitivity to photoperiod, is the 10hr photoperiod condition that received a heat shock after short chilling duration and whilst in forcing conditions, but before any buds had flushed yet. Because of this treatment, bud burst occurred earlier than expected (see Figure 2.9 A and Figure 2.12 B) and although this condition was omitted from the analyses, any significant effect that would have been present might have been levelled out by the removal of this condition.

Where interactions between photoperiod and chilling were significant, long photoperiods had an effect on time to bud burst after short chilling durations, reducing the number of days to bud burst. However this effect could not fully substitute for an incomplete fulfilling of the chilling requirements and disappears altogether when chilling requirements are completely fulfilled. This conclusion follows previous findings such as in *B. pubescens* and *B. pendula* clones (Myking and Heide 1995), and in Norway spruce (Søgaard et al. 2008), and is an

interesting finding that provides evidence for temperature importance in the current debate about environmental drivers of tree phenology (Chuine et al. 2010, Körner and Basler 2010).

2.4.4 Conclusions

In this study, very different responses in the timing of dormancy release were observed between two species subjected to the same environmental conditions. Both *B. pubescens* and *P. tremula* are considered to be opportunistic pioneering species that can be fast growing when an opening appears in a forest, although aspen trees prefer wetter soils than birch trees. The variation in responses between these two species suggest a slight nuance in the hypothesis that climate change affects the phenology of early-successional tree species more than that of late successional species (Caffarra and Donnelly 2010, Körner and Basler 2010) and that a thorough understanding of the effects of chilling duration but also of different chilling temperatures is needed on a species level and for more species than have been studied thus far.

The responses for multiple species need to be known in order to make meaningful decisions for conservation and management of forests and ecosystems. Indeed, a problem pointed out by Körner (2006) was the paradox that is created by mild winters as an effect of climate change: on the one hand, spring development may be delayed for some species because of insufficiently fulfilled chilling requirements, or on the other hand, bud burst may occur earlier in other species with low chilling requirements and low photoperiod-sensitivity (e.g. Cannell and Smith 1986).

Since the photoperiod effect was weaker for longer chilling durations, the possible effects of a climatic warming include earlier bud burst and therefore a higher risk of frost damage on newly emerged buds. This would be a particularly unfavourable trend for birch, with an earlier bud burst compared to aspen and with an ability to reach bud burst even after incomplete chilling, short photoperiods and a forcing temperature of 10°C. Indeed, *B. pubescens* has already shown a highly variable start of growing season over the decade 1981-1990,

compared to other species in the IPGs (Caffarra and Donnelly 2010). The risks for *P. tremula* may lie in an extended period of dormancy release or even a failure to reach dormancy release due to milder winters, leading to a loss of competitive advantage due to a fast growth that is characteristic for aspen. Many other forest species are likely to have a similar response to chilling, and models predicting the impacts of climate change on a large scale should not only consider earlier bud burst, but also take a possibility of delayed bud burst into account for some species. A delay in spring phenology was indeed detected in the field using NDVI (Normalised Difference Vegetation Index) on the Tibetan Plateau as a result of a slow fulfilling of chilling requirements (Yu et al. 2010).

2.4.5 *Suggestions for future research*

(1) Future experiments should take some limitations of the experiments described here into consideration. Several unforeseen difficulties were met during the experiment, causing the number of trees per treatment to be limited (five and eight, respectively, due to the propagation failure and trees available), and the forcing temperature to be relatively low in Experiment 1 (10°C, due to growth chamber failure). The response of bud burst to many different forcing temperatures and the interactions with other environmental factors is complicated and needs more investigation, certainly since it is one of the variables relevant to climate change impacts. An experimental design using more trees in a single condition would yield a stronger basis for conclusions.

(2) Clones were used in this experiment to rule out intra-specific variability as a factor of bud burst variation. These types of experiments using clones are very useful for modelling purposes, however in order to generalise these findings for forest ecosystems, studies evaluating more species and different genotypes and provenances per species are needed.

(3) As vegetative tree buds go through several phases of dormancy release, invisible to the naked eye but happening on a microscopic or molecular level, bud developmental studies are needed to fully understand the influence of environmental factors on dormancy release.

These observations would greatly improve existing phenological models (see for instance Sutinen et al. 2009 for an anatomical study on Norway spruce buds and Renaut et al. 2004 for a proteomic study on poplar buds).

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Chapter 3:

Modelling analysis of bud burst drivers in *B. pubescens* Ehrh. and

P. tremula L. in controlled environment experiments for use in process-based phenological models

3.1 INTRODUCTION

3.1.1 *The use and development of models in phenological research*

The modelling of phenological events is a natural follow-on after recording them, and has a long history starting in 1735 with Reaumur and his idea that the timing of phenological events could be estimated by accumulated daily temperatures from an arbitrary date (Chuine et al. 2003). Because an important aim of documenting the seasonality of life is to make future predictions, it is necessary to find the appropriate relationships between the driving factors and the observed phenomenon, even though the underlying mechanisms involved in the “perception” of those factors by plants are still not well understood (Sung and Amasino 2005). However, several recent insights in the molecular, physiological and biochemical pathways associated with dormancy induction and dormancy release of woody plants hold promise for more accurate predictions of phenological events in future (e.g. Renaut et al. 2004, Böhlenius et al. 2006, Ruttink et al. 2007, Sutinen et al. 2009, Santamaría et al. 2011).

Many phenological models have been developed to describe the timing of bud burst in perennial plants and many types of models have been used. Some bud burst models are based on experimental manipulations where one mechanism at a time was tested (e.g. Caffarra et al. 2011 a, 2011 b, Hänninen 1990, Murray et al. 1994, Sarvas 1972), while others have been developed using long-term datasets of natural populations or planted clones in phenological gardens (e.g. Kramer 1994, Chuine et al. 1999, Ibáñez et al. 2010). Besides the different types of observations, a distinction can also be made according to which factors are taken into account: in statistical models the timing of phenology is purely linked with climatic factors (e.g. the Spring Index model, Schwartz 1997), while in mechanistic models the relationships between environmental factors and biological processes within the plant are

included. An example of such a relationship is the 'chilling requirement' needed for the release of dormancy (Hänninen 1990). All these models have been built on each other and can therefore be considered as part of a family, in which one model is a special case of another one (for instance, when a parameter is considered as zero and left out of a basically similar equation). The trend is towards more complex models, yet not always yielding significantly more accurate predictions. Linkosalo et al. (2008) compared and tested some of these models (Thermal Time model and Unified model) with several other models and concluded that the simpler Alternating model performed best when tested with independent data of bud burst, while the use of more complex models such as the Unified Model (Chuine 2000) did not significantly change the prediction accuracy, due to over-parameterisation and random noise.

Tree phenology models are used in a variety of applications. For instance, Chuine and Beaubien (2001) established that bud burst models can be used to predict species range of deciduous trees (*Populus tremuloides* Michx. and *Acer saccharum* Marshall), which is essential information for species conservation and forest management. But also for the conservation of whole ecosystems it is important to be able to predict the timing of phenological phases of plants, which act as primary producers for a suite of organisms. Phenological models are therefore incorporated into dynamic global vegetation models (DGVMs) and forest productivity models (Cramer et al. 2001). Tree phenology also has an important effect on human health through the timing of allergenic pollen release into the atmosphere. In this instance, is not only the first onset of flowering that is important, but also the duration and the intensity of pollen release throughout the season of species such as *B. pendula* and *B. pubescens* (Linkosalo et al. 2010). Furthermore, models are used in the prediction of the impact of global warming on growing season changes of specific species (Cleland et al. 2007, Ibáñez et al. 2010), and on the increased risk of frost damage for temperate trees (Hänninen 2006).

3.1.2 *Brief description of some relevant bud burst models*

An overview of the most important models for this study is given here, leaving out several other very valuable models. The selection was made based on their advantages of simplicity (Thermal Time model, Alternating model) or their species specificity (Dormphot Model, based on the Unified Model).

3.1.2.1 Thermal Time model

The Thermal Time model, based on Reaumur's approach (Cannell and Smith 1983), is the most straightforward model of bud burst using only the sum of average daily temperatures as a predictor of bud burst in trees, when the temperature T is above a certain threshold temperature T_{crit} :

$$r(T) = \begin{cases} T - T_{crit} & T \geq T_{crit} \\ 0 & T < T_{crit} \end{cases} \quad (1)$$

where $r(T)$ is the rate of bud development as a function of T . The stage of bud development $S(t)$ at a certain time t , with t_0 the start date of temperature accumulation, is then according to Equation 2:

$$S(t) = \int_{t_0}^t r(T) dt = \sum_{t_0}^t r(T) \Delta t \quad (2)$$

Bud burst occurs when $S(t)$ reaches a threshold sum S_{crit} . This Thermal Time model was adapted into several different models taking dormancy into account, for instance in the Alternating model (Cannell and Smith 1983, Murray et al. 1989).

3.1.2.2 Alternating model

This model developed by Cannell and Smith (1983) assumes a negative exponential relationship (e.g. Figure 3.1) between the sum of forcing units (number of days with average temperature above a threshold of 5°C, also 'degree days') and the sum of chilling units

(number of days with average temperature below 5°C). This relationship is species-specific, and even between different species from the same genus the function can vary considerably (Figure 3.1). The critical temperature threshold depends on the accumulated chilling sum so that less forcing units are needed to reach bud burst when there is more chilling.

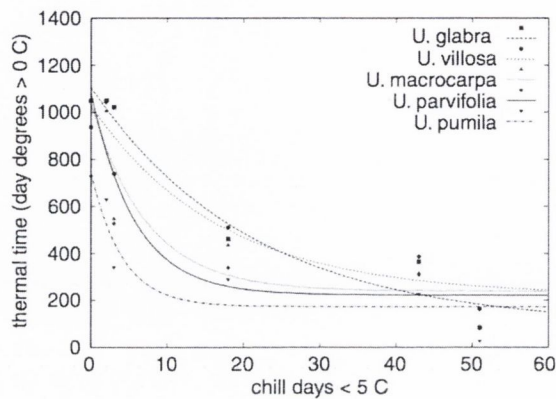


Figure 3.1 Negative exponential relationship between time to bud burst and exposure to chilling days below 5°C for five elm species (*Ulmus* spp.), from Ghelardini et al. 2010.

The threshold for $S(t)$ is described by the following function:

$$S_{crit}(t) = a + be^{cn(t)} \tag{3}$$

where $n(t)$ is the number of accumulated chilling days at day t , and a , b and c are model parameters.

3.1.2.3 Unified model

The Unified model developed by Chuine (2000) is a more complex model, unifying some of the assumptions of previously developed models, with main differences compared to the Thermal Time and Alternating models (a) that the start date of temperature accumulation does not start at an arbitrary date, but rather when a sufficient amount of chilling (following a sigmoid function, Equation 4) has occurred, and that (b) temperature sum accumulation also follows a sigmoid function (Equation 4).

$$\frac{1}{1 + e^{a(x - c)^2 + b(x - c)}} \tag{4}$$

Previous models (e.g. Cannell and Smith 1983, Hänninen 1990, Kramer 1994) can be considered as special cases of the Unified model; for instance the accumulation of chilling units follows a bell-shaped curve in the Unified model (with the top of the curve corresponding to the most optimal chilling temperature), as opposed to the more straightforward combination of two linear functions (Hänninen 1990, Figure 3.2). The critical threshold for bud burst in the Unified Model is described by a similar negative exponential function as in the Alternating model (Equation 3), in which parameter $a = 0$.

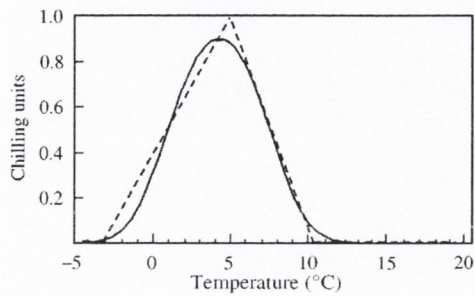


Figure 3.2 Two functions describing the rate of chilling development in trees as a function of temperature, with 5°C as the optimal temperature: (---) Alternating model (Hänninen 1990), and (—) Unified model (Chuine 2000). Figure from Chuine 2000.

3.1.2.4. Dormphot model

The Dormphot model was recently developed by Caffarra et al. (2011 a, 2011 b) as a variation on the Unified Model, but including experimentally determined relationships between photoperiod and temperature for *B. pubescens*. The rate of forcing and the rate of chilling are described by modified versions of the Unified Model, while also the rate of dormancy induction is included, as a function of daily temperature and photoperiod.

3.1.3 Aims of this study

Phenological models attempt to simulate the natural phenomenon of bud burst using the current knowledge of factors involved. Since it is not beneficial to over-complicate models with more and more parameters (e.g. Linkosalo et al. 2008), it is therefore all the more important to understand the importance of factors involved and understand how they influence phenological events.

In the previous chapter, the statistical significance of the factors (chilling duration and photoperiod length) influencing the experimental bud burst results in *Populus tremula* (European aspen) and *Betula pubescens* (downy birch) was determined using several statistical tests, such as a mixed effect ANOVA. In brief, chilling duration proved to have a significant effect on the timing of bud burst in all cases and photoperiod length had an effect on the timing of bud burst through an interaction factor with chilling duration in most cases. Here, the underlying causes of how these factors affected the timing of bud burst are identified by two means:

(1) By the novel approach of investigating the effect of (a) chilling duration and (b) photoperiod length on distribution factors, namely the skewness, kurtosis and coefficient of variation of the frequency distributions of the number of days to bud burst in each experimental condition, for *B. pubescens* and *P. tremula*. Expected results are higher kurtosis, lower variation and skewness closer to zero for plants that are chilled for longer and received a longer photoperiod as these plants received the more 'optimal' conditions.

(2) By fitting the results from the growth chamber experiments into an extensively used and well-tested model of bud burst: the Thermal Time model (Cannell and Smith 1983, Murray et al. 1989, Hannerz et al. 2003, Ghelardini et al. 2009, Harrington et al. 2010) for both species.

3.2 MATERIALS AND METHODS

3.2.1 Experimental data

3.2.1.1 Plant material

All *Betula pubescens* (birch) and *Populus tremula* (aspen) plants used in the experiment were clones to eliminate genotypical variability. The birches used in the experiments were the juvenile trees (height between 30 and 45 cm) that were propagated in 2003 and 2004 by Caffarra and Donnelly (2010) and kept outdoors in 1l plastic pots at the Trinity College Botanic Gardens, Dublin and subsequently at Teagasc Horticultural Centre in Kinsealy, (Co.Dublin). The aspen trees were commercially cloned juvenile trees purchased from Cappagh nurseries (Aughrim, Co. Wicklow, Ireland; EU Plant Passport 7238). For the experiments, the 3 to 4-year old trees with height between 80 and 120 cm were transplanted into plastic pots with diameter of 15-20 cm containing John Innes No. 3 compost, and were kept at Teagasc Horticultural Centre in Kinsealy. The average viable number of buds per aspen tree was 59, ranging between 24 and 152 buds per tree, and the average number of buds for birch trees was 16, ranging between 4 and 41 buds per tree.

3.2.1.2 Experimental design

Two experiments investigating dormancy release were conducted on aspen and birch trees to determine the effects of photoperiod and chilling requirement on the timing and percentage of budburst. All plants received natural chilling in ambient conditions and were introduced to experimental conditions at different times (2 different times in Experiment 1 and 3 different times in Experiment 2), giving each treatment a different chilling duration. Furthermore, different photoperiod conditions (8, 12 and 16 hours of daylight for Experiment 1, and 10 and 16 hours for Experiment 2) were applied to the plants in growth chambers. See section 2.2.2 and Table 2.1 for the details of the experimental procedures.

3.2.2 Analyses and fitted functions

Skewness, kurtosis and coefficient of variation are traits of the frequency, probability or distribution function. This function represents the frequency (or normalised frequency for distribution functions) of bud burst occurrences within a single tree at a given day after transfer into experimental conditions. The analysis performed for determining the effects of chilling duration (*Chilling*) and photoperiod length (*Photoperiod*) on the skewness, kurtosis and the coefficient of variation of the frequency distribution of the number of days to bud burst (variables respectively *Skewness*, *Kurtosis* and *CV*), was a standard univariate ANOVA (Analysis of Variance), executed using SPSS 18.0 and R 2.13.1. The skewness of a distribution is a measure for the asymmetry of the distribution curve, or the deviation of the mean from the mode. The value can be negative (left-skewed or with a longer tail to the left), zero (no skew), or positive (right-skewed or with a longer tail to the right). The kurtosis is a measure of 'steepness' of the curve, or of the fraction of the variance that is explained by infrequent extreme values of the variable compared to frequent deviations from the mean. Also the kurtosis can be indicated by a negative value (platykurtic, many deviations from the mean), zero (deviation as expected from a normal distribution) or by a positive value (leptokurtic, few extreme deviations from the mean), as depicted in Figure 3.3. Finally, the coefficient of variation is the normalised measure of variation around the mean, calculated as the ratio of the standard deviation to the mean and expressed as a percentage.

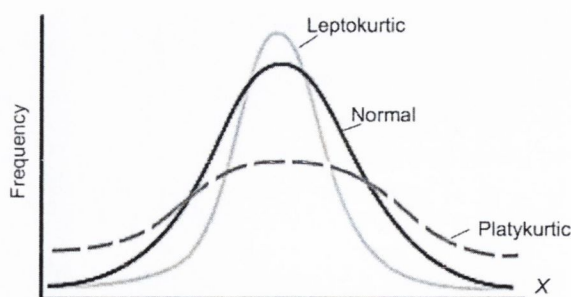


Figure 3.3 Representation of distribution curves with positive kurtosis (leptokurtic, grey curve), zero kurtosis (normal distribution, black curve) and negative kurtosis (platykurtic, dashed curve), from Miller-Keane and O'Toole (2005).

The assumptions for ANOVA analysis were tested and confirmed:

1. The normality of the data was tested using Q-Q plots. Counter intuitively, the variable *Skewness*, a measurement of deviation from a normal distribution of days to bud burst for each tree, was normally distributed. However, *Kurtosis* and *CV* had skewed distributions. Therefore, two separate transformations were applied to these variables: (1) a lognormal transformation for *CV*, and (2) the square root of *Kurtosis*, to which a value of 3 was added in order to make all values of *Kurtosis* positive before taking the square root. The transformed variables were respectively named *ln(CV)* and *S_Kurt*.
2. Levene's tests were conducted in order to test the null hypothesis that the error variance of the dependent variable was equal across groups. Homogeneity of variance was confirmed unless mentioned otherwise.
3. Independence of the samples was ensured by randomisation in the choice of plants with different heights and number of buds for each treatment. Although bud burst events were recorded several times on a single tree, the variables were calculated per tree and therefore observations between different trees could be considered as independent.

The function fitted using the experimental results from Chapter 2 was the Thermal Time model (Equation 3) for *P. tremula* and *B. Pubescens*, and chilling units were culculated using Equation 4 for chilling accumulation, as in the Unified model and Dormphot model. This model was chosen because it is simple, practical and still widely used (e.g. Hannerz et al. 2003, Bailey and Harrington 2006, Ghelardini et al. 2009, Harrington et al. 2010, Man and Lu 2010). Curve fitting was performed in SPSS 18.0 statistical software and R 2.13.1, using the *nls* package in R for non-linear models.

3.3 RESULTS

3.3.1 The effect of chilling duration and photoperiod length on the probability distribution of bud burst

In order to understand how the effects of chilling duration on the timing of bud burst were created, several parameters of the probability distribution of bud burst date (skewness, kurtosis, coefficient of variation) were subjected to one-way ANOVAs.

Firstly, the distributions of days to bud burst were created within each tree separately, and the mean bud burst dates per tree were analysed. Here, each data point of days to bud burst represents the average of one tree. The density plots in Figure 3.4 A and 3.4 B show the distribution of the mean bud burst dates of all trees in Experiment 2 (*B. pubescens* in Figure 3.4 A and *P. tremula* in Figure 3.4 B). From the steepness of the grey curves in both panels, the plots suggest a higher kurtosis of the density curves for longer chilling durations in both species, compared to shorter chilling periods (dotted and black curves, Figure 3.4 A and B). Furthermore, greater variation around the mean can be expected for shorter chilling durations compared to conditions with the longest chilling time, shown by the wider basis of the black curves in Figure 3.4.

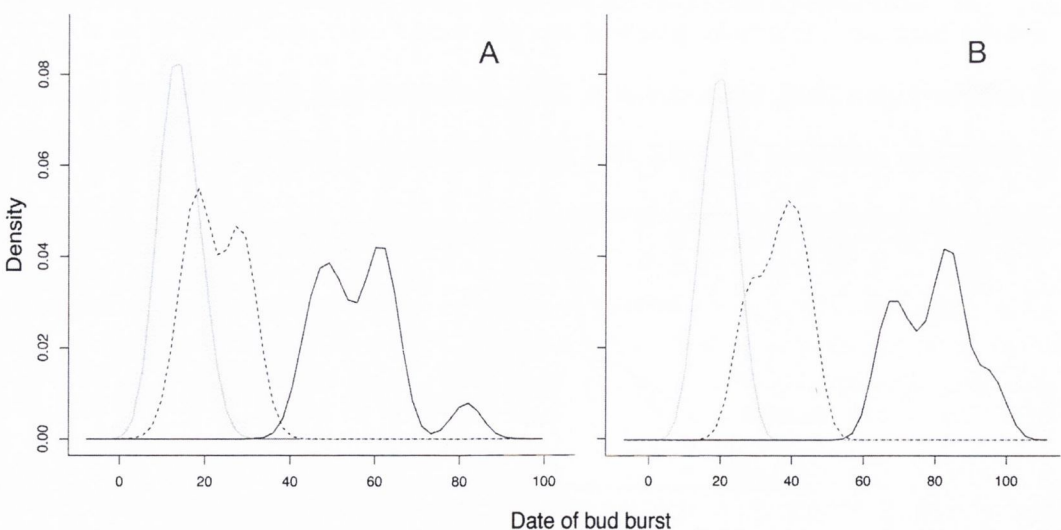


Figure 3.4 Density plots of mean bud burst date per tree in *Betula pubescens* (A) and *Populus tremula* (B) in Experiment 2. Grey plots: experimental condition with longest chilling duration (76 days), dotted plots: intermediate amount of chilling (53 days), and black plots: shortest chilling duration (25 days).

The density plot characteristics are summarised in Table 3.2. The trends from the plots in Figure 3.4 are however not very clear from the numerical results. In general, there is a tendency towards lower standard deviations for longer chilling periods (with a few exceptions, e.g. for the shortest chilling duration under 16hrs of daylight for *B. pubescens* in Experiment 2). However, when the standard deviation is normalised over the mean value of bud burst date in the coefficient of variation (CV), this trend becomes obscured. For *Kurtosis* and *Skewness*, no trends are evident from Table 3.2. Transformations are not considered in this analysis, because only the exact values of the variables from distributions of the mean bud burst values (as in Figure 3.4) are compared rather than averages.

Table 3.2 Average days to bud burst (BBD, $\pm$ standard variation), *Skewness*, *Kurtosis* and *Coefficient of Variation* (CV) of days to bud burst distribution curves, for *Betula pubescens* in Experiment 1 and 2, and for *Populus tremula* in Experiment 2. Experimental conditions were created by using different chilling durations (*Chilling*) and photoperiod lengths (*Photo*). *Heat shock treatment (one day at 40°C during forcing)

Chilling (days)	Photo (hrs)								
		BBD (days)	Skewness	Kurtosis	CV	BBD (days)	Skewness	Kurtosis	CV
<i>Betula pubescens</i>						<i>Populus tremula</i>			
Experiment 2									
25	16	60.5 ± 3.4	-1.2	1.9	5.7	80.4 ± 10.7	0.1	-0.3	13.3
25*	10*	52.5 ± 13.2	2.4	6.1	25.1	70.9 ± 21.9	-1.7	3.4	30.9
53	16	17.6 ± 2.0	-1.7	3.5	11.1	31.1 ± 4.7	0.5	-1.7	15.0
53	10	28.5 ± 1.9	0.6	1.6	6.8	41.4 ± 3.2	-0.5	-0.6	7.8
76	16	14.6 ± 3.8	0.2	-1.7	26.2	18.4 ± 2.9	0.1	-1.5	15.7
76	10	13.4 ± 2.4	2.0	4.3	17.6	20.8 ± 3.5	-1.0	0.9	16.8
Experiment 1									
35	16	76.8 ± 10.1	1.1	0.7	13.1				
35	12	96.8 ± 9.6	-0.7	-1.2	9.9				
35	8	94.1 ± 16.0	0.3	-2.3	17.0				
82	16	23.6 ± 6.8	1.8	3.4	28.8				
82	12	24.5 ± 1.2	2.2	4.8	4.8				
82	8	27.9 ± 6.1	0.7	-0.9	21.7				

In the second section of the analysis, the three distribution variables *Skewness*, *Kurtosis* and *CV* were calculated separately for each distribution function of bud burst per tree (Figure 3.5). Within each of the distributions, each data point of days to bud burst represents a single bud in the particular tree, while in the analysis each data point of a variable represents one tree.

After calculating the variables, transformations were performed on the resulting data of *Kurtosis* and *CV* in order to normalise the data and subsequently the ANOVAs were carried out using the transformed data (Table 3.3).

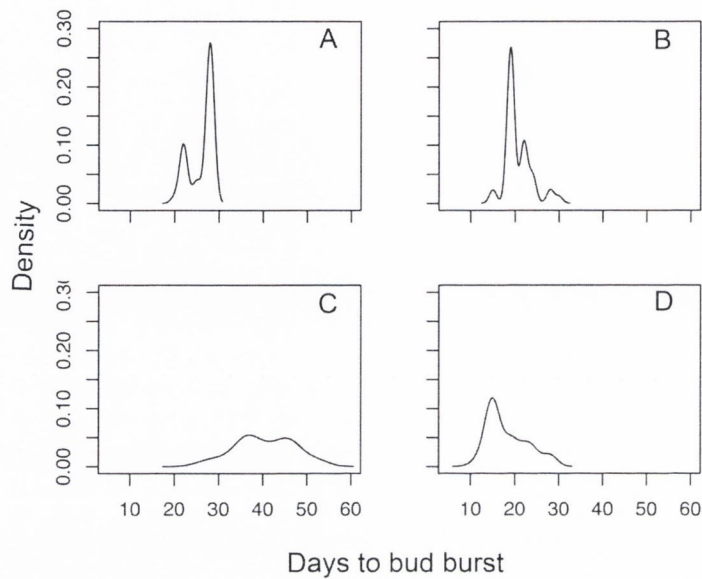


Figure 3.5 Distribution curves of bud burst dates for four single *Populus tremula* trees in Experiment 2 under different photoperiod conditions (tree A and B: 16 hours of daylight; tree C and D: 10 hours) and different chilling conditions (tree A and C: chilling duration of 53 days; tree B and D: 76 days of chilling).

The graphs in Figure 3.5 are examples of the resulting shape variations of the distribution curves of bud burst per tree. These variations in shape are reflected in the varying standard deviations of *Skewness*, *Kurtosis* and *CV* in Table 3.3. In Experiment 1, no significant effects of chilling duration or photoperiod length on either of the variables tested could be confirmed. For Experiment 2, a significant effect of chilling duration on $\ln(CV)$ was found in *B. pubescens*, on S_Kurt in *B. pubescens* and *P. tremula*, and on *Skewness* in *P. tremula*. Although in the latter test the equality of variances was not validated with Levene’s test, the *p*-value is convincingly low (<0.001) and indicated by (!) in Table 3.3. No significant effects of photoperiod length were discovered. The influence of photoperiod length on the distribution variables was not tested for *B. pubescens* in Experiment 2, since no significant main effects of photoperiod were detected on bud burst date for this experiment (see Table 2.10).

Table 3.3 Average values ($\pm$ standard deviation) of variables *Skewness*, *Kurtosis* and *CV* (coefficient of variation) and of the transformed variables *S_Kurt* and *ln(CV)* of distribution functions of days to bud burst (*BBD*) per tree in different experimental conditions for *B. pubescens* and *P. tremula*; and one-way ANOVA results of the effects of chilling duration (*Chilling*) and photoperiod length (*Photo*) on the variables *Skewness*, *S_Kurt* and *ln(CV)*. Bold *p*-values are significant on a 0.05 level.

		Average and standard deviation						ANOVA								
Chilling (days)	Photo (hrs)	BBD	Skewness	Kurtosis	S_Kurt	CV	ln(CV)									
		(days)						Skewness	S_Kurt	ln(CV)						
<i>Betula pubescens</i>																
Experiment 1																
35	16	76.8 ± 10.1	-1.9 ± 0.7	4.7 ± 3.3	2.7 ± 0.6	6.4 ± 2.8	1.8 ± 0.5	Chilling	F	0.23	0.02	0.12				
35	12	96.8 ± 9.6	-0.8 ± 1.0	0.2 ± 2.7	1.6 ± 0.8	15.9 ± 2.2	2.8 ± 0.1		p	0.64	0.99	0.73				
35	8	94.1 ± 16.0	-0.9 ± 0.9	0.4 ± 3.4	1.6 ± 1.0	9.0 ± 0.7	2.2 ± 0.1	Photo	F	1.23	1.00	0.74				
82	16	23.6 ± 6.8	-0.7 ± 1.2	1.0 ± 4.6	1.7 ± 1.2	12.8 ± 7.0	2.4 ± 0.5									
82	12	25.6 ± 1.2	-1.7 ± 0.6	3.5 ± 2.8	2.5 ± 0.6	8.0 ± 3.6	2.0 ± 0.4						p	0.31	0.38	0.48
82	8	28.0 ± 6.1	-0.4 ± 1.0	0.1 ± 2.5	1.7 ± 0.6	9.6 ± 4.6	2.2 ± 0.5									
Experiment 2																
25	16	80.4 ± 10.7	-0.2 ± 0.9	0 ± 2.1	1.6 ± 0.6	13.2 ± 3.5	2.5 ± 0.3	Chilling	F	0.36	3.33	15.80				
25*	10*	78.3 ± 10.4	-0.5 ± 1.1	1.2 ± 2.4	1.9 ± 0.5	18.9 ± 5.1	2.9 ± 0.3						p*	0.70	0.41	<0.001
53	16	31.1 ± 4.7	-0.1 ± 0.8	-0.7 ± 1.1	1.4 ± 0.3	20.1 ± 9.9	2.8 ± 0.5	Photo	F	0.00	1.25	0.00				
53	10	41.5 ± 3.2	0.1 ± 0.3	-0.3 ± 0.4	1.6 ± 0.1	16.2 ± 4.9	2.7 ± 0.3									
76	16	18.4 ± 2.9	0.9 ± 3.5	0.9 ± 0.9	1.9 ± 0.2	16.4 ± 5.5	2.7 ± 0.3						p	0.96	0.27	0.96
76	10	20.8 ± 3.5	0.8 ± 0.4	0.9 ± 1.8	1.9 ± 0.4	14.4 ± 4.8	2.6 ± 0.3									
<i>Populus tremula</i>																
25	16	80.4 ± 10.7	-0.2 ± 0.9	0 ± 2.1	1.6 ± 0.6	13.2 ± 3.5	2.5 ± 0.3	Chilling	F	9.89	4.21	0.54				
25	10	78.3 ± 10.4	-0.5 ± 1.1	1.2 ± 2.4	1.9 ± 0.5	18.9 ± 5.1	2.9 ± 0.3						p	<0.001	0.02	0.59
53	16	31.1 ± 4.7	-0.1 ± 0.8	-0.7 ± 1.1	1.4 ± 0.3	20.1 ± 9.9	2.8 ± 0.5	Photo	F	0.00	1.25	0.00				
53	10	41.5 ± 3.2	0.1 ± 0.3	-0.3 ± 0.4	1.6 ± 0.1	16.2 ± 4.9	2.7 ± 0.3									
76	16	18.4 ± 2.9	0.9 ± 3.5	0.9 ± 0.9	1.9 ± 0.2	16.4 ± 5.5	2.7 ± 0.3						p	0.96	0.27	0.96
76	10	20.8 ± 3.5	0.8 ± 0.4	0.9 ± 1.8	1.9 ± 0.4	14.4 ± 4.8	2.6 ± 0.3									

* heat shock condition not included in ANOVA analysis (!) Equality of variances not guaranteed

Post-hoc tests (Tukey's tests) indicated that the significant effects implied a higher coefficient of variation for longer chilling durations for *B. pubescens*, while for *P. tremula* longer chilling durations resulted in a more positive skewness, with distributions skewed further to the right. For *Kurtosis*, a significantly higher value, meaning steeper distribution curves was found for plants that were fully chilled, however for the shorter chilling durations the difference in kurtosis was minor.

3.3.2 *Modelling analysis for bud burst*

The thermal time to bud burst follows an inverse exponential curve according to the Thermal Time model described in Equation 3. The experimental data of both exepriment seasons were combined into one single dataset per species, resulting in five different conditions of chilling

duration: i.e. 25, 35, 53, 76 and 82 days of chilling in ambient conditions, with an average daily temperature under 10°C, before the plants were subjected to experimental conditions. The values from Experiment 1 at 35 chilling days (not considered in the analysis in Chapter 2) is added here, because a 'correction' was made for the heat shock the plants received, by calculating the thermal time received by the plants. Accumulation of thermal time was defined as day degrees above 0°C, starting from 1st January or from the first day the plants were transferred into experimental conditions. After calculating the average thermal time to bud burst per chilling condition, the exponential model of Equation 5 was fitted using the resulting data (Figure 3.5). The fitted curves for both species did not show much difference in shape or decline, although thermal time decreased slightly faster for *P. tremula* than for *B. pubescens* for the lower chilling durations (leftmost area of the graph) before levelling off simultaneously towards an asymptotic value, not shown on the graph but represented by the parameter *a* in Equation 3 and Table 3.4. However, it is clear from Figure 3.5 that *B. pubescens* has a lower chilling requirement than *P. tremula*, which was already suggested in Chapter 2: the curve describing the timing of bud burst is for all chilling conditions lower for *B. pubescens* than for *P. tremula*.

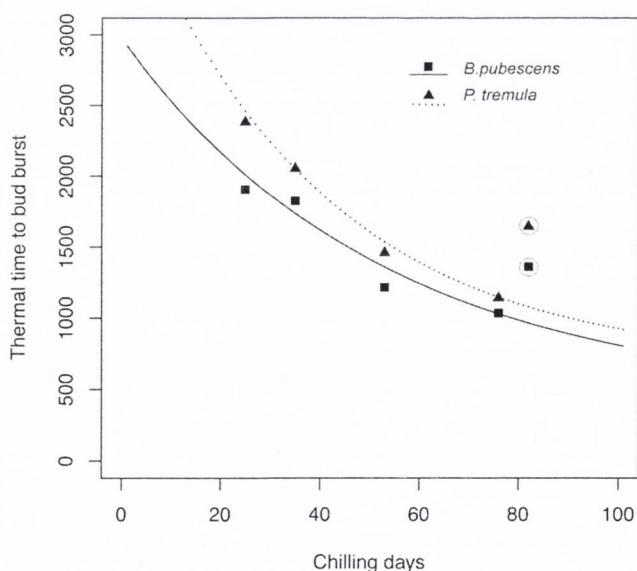


Figure 3.5 Thermal time to bud burst (day degrees >0°C from January 1st) as a function of chilling exposure duration (chilling days <10°C in ambient conditions since 1st September) in *Betula pubescens* and *Populus tremula* in two separate experiments. The fitted functions are Equation 5 (*B. pubescens*) and Equation 6 (*P. tremula*). The circled points were excluded from the fitting line.

Since different forcing temperatures were used in Experiment 1 (10°C) compared to Experiment 2 (20°C), some variation in response to chilling duration between the two experiments was expected, since this simple model does not take forcing temperature into account. Indeed, for the condition with longest chilling duration in Experiment 1 (82 days), the thermal time to bud burst was aberrant compared to the responses of the other conditions for both species. Despite the correction for the low forcing temperature through the thermal time calculations, 10°C (on the border of chilling-forcing) was not a sufficient forcing temperature in order for these buds to flush as early as predicted by this model, although significantly earlier than for the buds with partial chilling (35 days) under the same forcing temperature. Therefore, these data points were omitted from the curve fitting analysis (circled points in Figure 3.5). The parameters resulting from the curve fitting are shown in Table 3.4 for each species, while Equation 5 (*B. pubescens*):

$$y = 430 + 249e^{-0.019x} \tag{5}$$

and Equation 6 (*P. tremula*):

$$y = 672 + 3340e^{-0.026x} \tag{6}$$

with y= thermal time and x= chilling days, describe the functions that are most efficiently fitting the experimentally determined bud burst data, with an explained variance percentage of repectively 94 and 99%. Parameter *a* is the asymptotic value of the curve, and represents the estimation of the thermal time to bud burst after (theoretically) infinitely long chilling durations, according to this model.

Table 3.4 Parameter values (*a*,*b* and *c*) for the relationship between thermal time to bud burst (day degrees >0°C since 1st January or since transfer into experimental conditions) and chilling days (<5°C), as expressed in Equation 3, for each species studied. e.v. = explained variance.

Species	<i>a</i>	<i>b</i>	<i>c</i>	e.v. (%)
<i>B. pubescens</i>	430.3	2492.4	-0.019	94.2
<i>P. tremula</i>	671.9	3339.9	-0.026	99.5

3.4 DISCUSSION

3.4.1 *The effect of chilling duration on the probability distribution of bud burst*

Bud burst models are mathematical attempts to estimate the timing of bud burst as accurately as possible, for the purpose of forestry or agriculture. Most of these models take external factors into account, such as temperature and photoperiod. However, the internal processes that are occurring within a single tree and within a single bud can also significantly influence the date of bud burst.

This is the first time variability of bud burst has been statistically described for use in phenological models. From an evolutionary point of view, it is advantageous to keep the variation in timing of bud burst to a minimum when environmental conditions are good and for the buds to flush simultaneously, however this does not always occur due to genetic, physiological or other environmental constraints. The inclusion of variation factors into phenological models would greatly improve the predictive power of these models. Traditionally, phenological models have used a static value for date of bud burst, calculated by averaging the data. However, given the fact that phenophases usually occur over an extended period of time, the predictive accuracy of these models can be enhanced by having a dynamic component of pheno-phase to facilitate this variability.

This study has indicated that the investigation of the effects of environmental triggers on distribution factors for the use of modelling bud burst is a novel and applicable approach. For instance, the significant effect of chilling duration on the skewness of bud burst distributions within a single aspen tree, suggests that bud burst for insufficiently chilled trees is a slower process in the beginning, with fewer buds opening straight after first bud burst, and only pick up later on when more buds open. Bud burst within a single aspen tree followed a negatively skewed distribution in this case, with a tail towards the left and a peak towards the right (Figure 3.6 A). On the other hand, after longer exposure to chilling, bud burst followed a different time pattern in *P. tremula*: the skewness of the distribution was more positive, with a tail towards the right and a peak towards the left (Figure 3.6 B), suggesting the majority of

buds flushed early on, while the remaining buds opened more sparsely. This trend was also visible in Figure 3.5, where distributions of B and D (with 76 chilling days) showed high peaks that were more inclined towards the left compared to trees A and C in Figure 3.5, with 53 chilling days. A different way of interpreting these results is that the mean values of bud burst did not simply move towards earlier dates after longer exposure to chilling (see Chapter 2), but also the majority of buds, represented by the curve peaks in Figure 3.6, flushed earlier in trees that had undergone longer chilling, resulting in a positive skewness (Figure 3.6 B).

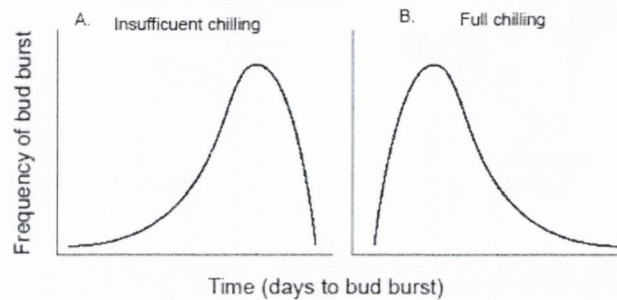


Figure 3.6 Schematic representation of the chilling effect on the distribution of bud burst in time within a single *P. tremula* tree.

Furthermore, the effect of chilling duration on the coefficient of variation in *B. pubescens*, suggested that a longer chilling exposure leads to a more concentrated occurrence of bud burst on a tree, while in insufficiently chilled trees flushing is more distributed. Both the results for distribution skewness and coefficient of variation can be explained from an evolutionary point of view. Within populations, and even within genotypes as shown in this study, there is a great variation in timing of bud burst (Rousi and Heinonen 2007). This variation in bud burst date could be explained by an evolutionary strategy to avoid a synchronic diminution of foliage when there is a peak in abundance of insect herbivores (Singer and Parmesan 2010).

Skewness and kurtosis parameters are used frequently in synchrony studies and shows potential to also learn more about the processes within plants during the dormancy- and dormancy release cycle. For example, caterpillars feed on newly emerging leaves and subsequently birds feed on the caterpillars. So in order for an ecosystem to survive, the timing of these interdependent phenophases must remain synchronised. This example shows that it is necessary to examine ecosystems as a whole (Giménez et al. 2011).

The coefficients of variation and measures of skewness in the frequency distribution of bud burst of *Pseudotsuga menziesii* (Mirb.) Franco (Douglas fir) were also taken into consideration in the study of Jermstad et al. (2001), however these were used for the purpose of QTL mapping (Quantitative Trait Loci). Jermstad et al. (2001) found that terminal bud flush in general followed a normal distribution, while lateral buds preceded terminal buds in flushing date, which produced negatively skewed distributions for bud flush (resulting in a tail to the left). This study however did not distinguish between terminal and lateral buds, as juvenile poplar trees usually have a long stem with many lateral buds and without many side branches, and the birch trees did not have enough buds to analyse lateral and terminal buds separately.

3.4.2 Fitting of the experimental data to a mechanistic model

The parameters from the fitted thermal time functions described the data well, with 94 and 99% of the variance explained by the models for *B. pubescens* and *P. tremula*, respectively. The values of the parameters are in line with previous studies (e.g. Ghelardini et al. 2009 for elm species and Harrington et al. for Douglas-fir), and Murray et al. (1989) also found a lower chilling requirement for a birch species (*B. pubescens*) compared to a poplar species (*P. trichocarpa*). Many studies use different start dates and different temperature thresholds. This is not surprising, since different species respond differently, however the use of these thresholds is not consistent. Harrington et al. (2010) compared models with different start – and stop dates for accumulation of chilling and forcing for the prediction of bud burst in Douglas fir. They suggested that chilling and forcing are not two separate functions and might be operating at the same time. This view is not yet understood at the molecular level, but it is likely that systems (e.g., different genes or transcription factors, or epigenetic effects) can overlap temporally (perceive and integrate both chilling and forcing) and also interact to result in resumption of cell division in the buds (Sung and Amasino 2005, Harrington et al. 2010). Similarly to Linkosalo et al. (2008), Harrington et al. (2010) concluded that simple, universal functions should be used for modelling the effectiveness of temperature for chilling and forcing, until a better understanding of physiological mechanisms in plants will help improve

existing models.

3.4.3 Conclusions

In conclusion, skewness (in *P. tremula*), coefficient of variation (in *B. pubescens*), and kurtosis (in both species), of the frequency histograms of days to bud burst were dependent on chilling duration. This study shows that bud burst date is more than a static mean value, and needs further study within populations and even genotypes, which are very frequent in natural conditions, particularly for the vegetatively reproducing aspen trees. Although bud burst phenology is widely studied between ecotypes, along gradients, or in phenological gardens, little is known about the within-population variation. Rousi and Heinonen (2007) concluded that due to the large inter-and intra-annual variation within *B. pendula* populations, bud burst estimates can only be reliable when measurements longer than a few decades are used for models. Also mismatches between insects and host-plants are proof that more research is needed on the tree-to-tree variation of bud burst and how climate change can affect this variation. Finally, it is recommended that this trait should be incorporated in bud burst models, such as the Dormphot model and tested whether the inclusion increases the predictability of bud burst in trees.

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Chapter 4:

Genetic variation in genes controlling dormancy-related traits in natural populations of European aspen (*Populus tremula* L.)

4.1 INTRODUCTION

4.1.1 *Adaptation of phenological responses in trees*

The limitation of tree growth at low temperatures to avoid cold injury is an important adaptation for survival in temperate regions, and is regulated by local environmental conditions. Because both survival and reproduction are affected by climate change, through the timing of bud burst and flowering, there are strong evolutionary pressures at play on these phenological traits (Hoffmann and Sgrò 2011). The number of recent reviews on adaptation, climate change and forest trees (e.g. Jump and Penuelas 2005, Parmesan et al. 2006, Aitken et al. 2008, Bradshaw and Holzapfel 2008, Donnelly et al. 2011) demonstrates an increased level of interest in the genetic basis of plant responses to the environment and that there is a need for a deeper understanding of the impact of climate change on adaptation in trees. The unique characteristics of trees such as wood formation, cold hardiness and leafing and flowering phenology cannot be studied using the annual herbaceous model plant *Arabidopsis thaliana*. Hence, a model system for forest trees was proposed, i.e. poplar (*Populus* spp.; Bradshaw et al. 2000). The genome of *Populus trichocarpa* Torr. and A.Gray, or black cottonwood has been fully sequenced by the International *Populus* Genome Consortium and extensive EST resources of this species have been made available (Tuskan et al. 2006). After *Arabidopsis thaliana* (L.) Heynh. (thale cress; The *Arabidopsis* Genome Initiative 2000) and *Oryza sativa* L. (rice; Goff et al. 2002), it was the third plant species to be fully sequenced and together with related *Populus* species, it has been used in numerous plant physiological and molecular studies (e.g. Jansson and Douglas 2007). Thus, the poplar genomics resources available provide the opportunity to identify and study the genes involved in phenology in trees.

4.1.2 The importance of genetics in tree phenological responses

Local adaptation is a result of the balance between natural selection and gene flow, and will occur if selection is the strongest component (Chaine 2000). It is generally assumed that plants are locally adapted to their environment, although a meta-analysis revealed that local adaptation in herbaceous plants depends strongly on population size and occurs significantly less commonly in smaller populations (Leimu and Fischer 2008). However, local adaptation in trees was reviewed by Savolainen et al. (2007), who found evidence in numerous publications for local adaptation along latitudinal and longitudinal clines. For instance, native Scots pine (*Pinus sylvestris*) populations from most parts of its range were grown in the same conditions at a common location. Those from local populations performed better in terms of height and survival compared to those transferred from populations farther away (Savolainen et al. 2007). Several common-garden studies have indicated that the genetic makeup of species and populations has a more important influence on bud burst than environmental factors. When trees from northern and southern populations are transplanted to a common environment, they mostly retain their phenotypic response of bud burst, showing that this trait is under strong genetic control and that the trees are locally adapted to their environment (Frewen et al. 2000, Howe et al. 2003). Bradshaw and Stettler (1995) even calculated using a mapping QTL (Quantitative Trait Loci) approach that up to 98% of the total phenotypic variance of bud burst in *Populus* hybrids (*P. trichocarpa* x *P. deltoides*) was explained by heritability (the proportion of variation in the phenotype due to genetic variation). They also found latitudinal and altitudinal trends in timing of bud burst.

Common garden studies and reciprocal transplant experiments are very useful as an indication of the importance of the genetic control of a particular trait. In order to study the underlying genetic variation in tree populations, candidate genes must be identified. Candidate genes are specific loci with a predicted biochemical function or with a homology to genes from other species, suspected of affecting the trait of interest (Taiz and Zeiger 2006).

4.1.3 The search for candidate genes involved in dormancy

Finding the link between complex traits such as bud burst and the different genes involved in this trait is not as straightforward in trees as in *Arabidopsis* for example, because of the long generation period of trees (Gailing et al. 2009). However, with comparison of whole genome sequences of both genera belonging to the rosid clade, putative *Populus* orthologues of *Arabidopsis* genes can be more readily identified (Brunner et al. 2004). These genes can then be targeted as potential candidates for further study.

Unknown genes may be identified and characterised through a combination of quantitative trait locus (QTL) analyses, gene expression studies, and genetic association mapping (Gailing et al. 2009). For instance, QTL mapping experiments and association studies in *Populus* have revealed genes in the flowering time network of annual plants that are also associated with flowering and vegetative bud development in trees (Frewen et al. 2000; Chen et al. 2002; Böhlenius et al. 2006; Ingvarsson et al. 2008; Ma et al. 2010). Association maps estimate the locations of genetic markers in the genome, which can be linked to a particular phenotype based on QTLs. Once the genes have been identified, variation within them can be assessed and putative associations determined. By comparing the genetic diversity between candidate genes and neutral markers, the level of local adaptation can be estimated. For instance, Ingvarsson et al. (2006) and Hall et al. (2007) have shown that adaptive population differentiation along a latitudinal cline has occurred in *P. tremula* *Phytochrome B2* genes across European populations, despite substantial gene flow levels (the proportion of new genes migrating into a population, Lowe et al. 2004). A number of candidate genes have been found for traits such as bud burst and dormancy release and these can be used to test for variation based on temperature clines. The gene regions used are described in detail in section 2.2.

4.1.4 Link between genetic pathways of flowering and dormancy control

As early as 1857, the capability of certain plants to flower in spring was attributed to a period of cold temperatures (vernalisation) to which they were subjected to during the winter (Klippart, in: Chouart 1960). Because of the similarity of this with a chilling period necessary for dormancy release, and the common key triggers (temperature and photoperiod) that play a role in both flowering and dormancy control, the hypothesis of a common signalling mechanism was suggested more than 50 years ago (Chouart 1960), but only more recently the molecular signalling processes are starting to be revealed (Horvath 2009, Lagercranz 2009). The role of phytochromes (PhyA and PhyB) in the perception of daylength and in bud dormancy has been known for longer (Howe et al. 1996, Frewen et al. 2000), but Böhlenius et al. (2006) provided the first evidence of shared molecular components which play a central role in the flowering pathway: overexpression of the poplar FT (FLOWERING LOCUS T) homologue PtFT1 did not only affect flowering in transformed *P. tremula* x *P. tremuloides* hybrids, but also acted as a suppressor of growth cessation and bud set. More examples are given in section 2.2, when the candidate genes used in this study are discussed. Another set of genes collectively named DORMANCY ASSOCIATED MADS-BOX (DAM) genes are very similar to each other, but differentially expressed during dormancy phases in raspberry (*Rubus idaeus* L.; Mazzitelli et al. 2007) and peach (*Prunus persica* (L.) Batsch; Bielenberg et al. 2008). Thus, many of the flowering genes identified in *Arabidopsis* have potential in phenological roles in trees.

4.2.5 Aims of this study

The aims of this particular work were to quantify the genetic variation of genes involved in the release of dormancy both within and between natural populations of *Populus tremula* and to investigate if clinal genetic variation along latitudinal and longitudinal gradients in Europe exists. The traits of interest were spring phenological traits such as bud burst and reactivation of growth after dormancy; and the genes of interest were linked with temperature. This study

seeks to analyse single nucleotide polymorphisms (SNPs) and patterns of DNA sequence variation in candidate genes between the different European populations in order to test:

- (1) what level of variation exists in a set of genes involved in phenological responses in trees
- (2) whether variation along a particular cline between the populations exists at the individual SNPs, which would suggest that an adaptive response to local conditions has occurred, and
- (3) whether there is evidence for variation from expected low genetic differentiation of the populations.

Low differentiation between-population is expected since levels of gene-flow is high in wind-pollinated and wind-dispersed species such as *P. tremula* (Ingvarsson 2005, Gailing et al. 2009). Differentiation of populations would suggest some form of isolation based on local adaptation. This novel study is the first of its kind to examine pan-European natural populations of European aspen and will have implications for forestry and conservation policy.

4.2 METHODS

4.2.1 Plant material

The plant material (*P. tremula*) for this study was collected between February 2009 and March 2010. Plant material from a broad latitudinal range across Europe was used in order to determine (1) clinal variations (adaptive divergence along a latitudinal and/or longitudinal gradient) of targeted genes across the geographical area examined, and (2) the diversity of these genes present within certain populations, showing the capacity of such populations to adapt to future climatic changes. A letter was sent in January 2009 to 464 botanic gardens across Europe, requesting twig samples from natural populations that contained at least 4 buds. It was emphasised that the samples should not be collected in a planted garden, but the expertise from the collectors was counted upon to search for populations as 'natural' as possible. 10 individual trees were requested per population, with sampled individuals at least 10m apart to avoid sampling the same clone. The response was successful as the target of 40 populations was reached, from a large geographic distribution (Table 4.1, Appendix 4.1). The samples were sent by post or brought back to the laboratory in the National Botanic Gardens (Glasnevin, Dublin) by the collectors and immediately after arrival the buds were removed from the twigs and stored in cryotubes in a -80 freezer until DNA extraction. The advantage of requesting bud samples rather than leaf material was that the bud scales surrounding the young leaf tissue kept the material fresh and suitable for DNA studies. When leaf samples were taken (Table 4.1, see Appendix 4.1 for the full list of samples collected), they were stored in silica gel for optimal dehydration and preservation of the plant material (Chase and Hills 1991). The scope of this project did not allow for all samples to be analysed, so a representative subset was chosen for the analysis, which included samples from a broad geographical range (see Table 4.1 and Figure 4.2). Per population, 5 individuals were sequenced. The priority was given to slightly more populations from a wide range rather than more samples per population. This decision meant it was possible to calculate nucleotide diversity but had implications for conclusions on population differentiation.

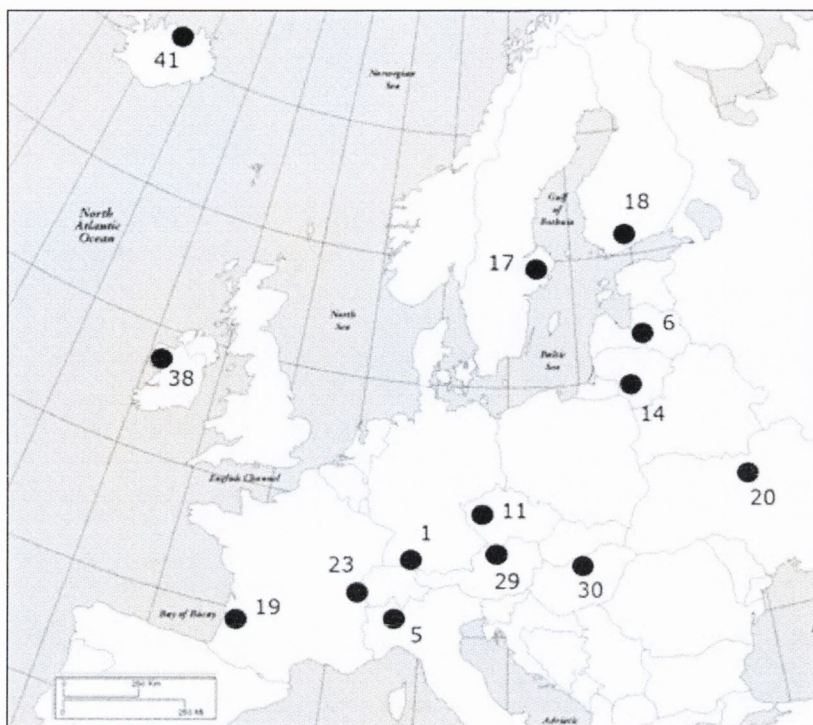


Figure 4.2 Map showing locations and population numbers of *P. tremula* material used in the genetic analysis (details in Table 4.1).

Table 4.1 Details of *P. tremula* material used in the analysis: populations and locations, and collection details.

Population number	Population name	Country	Latitude (°N)	Longitude	Altitude (m)	Collector	Date collected	Material type
1	Konstanz	Germany	47°38'	9°8'E	567	Gregor Shmitz	06-Feb-09	Buds
5	Lago Maggiore	Italy	45°59'	8°36'E	955	Caretti Franco	06-Feb-09	Buds
6	Jaunjelgava	Latvia	56°19'	25°20'E	80	Aija Liepkalne	03-Feb-09	Buds
11	W Bohemia	Czech Republic	49°44'	13°22'E	330	Vacvlava Peskova	Feb-09	Buds
14	Marijampol_	Lithuania	54°37'	23°17'E	70	Ar_nas Balsevi_jus	Feb-09	Buds
17	Uppsala	Sweden	59°51'	17°38'E	26	Bot. Gard. Uppsala Univ.	Feb-09	Buds
18	Elimäki	Finland	61°12'	24°00'E	80	Jaakko Saarinen	01-Mar-09	Buds
19	Bordeaux	France	44°50'	00°33'W	95	Laurent Lestourneaud	01-Mar-09	Buds
20	Kiev	Ukraine	51°28'	31°18'E	50	Mykyta Peregrym	15-Feb-09	Buds
23	Lausanne	Switzerland	46°17'	7°29'E	1048	Pascal Vittoz	14-Feb-09	Buds
29	Linz	Austria	48°18'	14°17'E	272	Ferdinand Lenglachner	12-Mar-09	Buds
30	Budapest	Hungary	47°31'	18°56'E	270	Attila Makay	01-Apr-09	Buds
38	Sligo	Ireland	54°14'	8°23'W	5	Annelies Pletsers	23-Aug-09	Leaves
41	Akureyri	Iceland	65° 51'	17°50 W	27	Heidi Salstad	Jul-09	Leaves

P. tremula is known to hybridise with related species; therefore taxonomic verification was an important issue. Twigs were checked for identification on arrival, although identification from limited samples of twigs can be difficult. Photographs were taken in case sample confirmation was required at a later date. During the DNA sequence analysis one population was

highlighted as being very different. This was shown to be a different species using a BLAST search (*P. alba* or white poplar in the case of population 7, see Appendix 4.1).

4.2.2 Candidate gene selection

The candidate genes were chosen based on recent scientific literature of genetic studies on dormancy-related traits in trees and other woody species. The genes of interest were mainly linked with temperature responses rather than photoperiod, and also with spring phenological traits such as the breaking of dormancy and reactivation of growth. This was a choice made since most research in this area is focused on the effects of photoperiod on cessation of growth whereas temperature-based mechanisms will be affected more in the face of climate change. The literature search included EST (Expressed Sequence Tags) analyses (e.g. Sterky et al. 2004), QTL (Quantitative Trait Loci) mapping studies (e.g. Cervera et al. 2001), and gene expression and transcription studies (e.g. Rhode et al. 2007). A search for selected gene regions of interest was performed in GenBank (NCBI; <http://www.ncbi.nlm.nih.gov>), and when *Populus* homologues of the candidate genes were found using BLAST (Basic Local Alignment Search Tool), the locations of the regions in the *Populus* genome were verified in specific databases such as PopGenIE (*Populus* Genome Integrative Explorer; Sjödin et al. 2009), the JGI *Populus trichocarpa* v1.1 Genome Browser (Joint Genome Institute, Tuskan et al. 2006), and the more recent version JGI v2.2 of the *Populus* genome incorporated in Phytozome v7.0 (Joint Genome Institute, <http://www.phytozome.net/poplar>). The *Populus* genome is made up of over 500 million base pairs, divided into 19 chromosomes. The genome browsers use the term 'linkage group', corresponding with chromosomes. Linkage groups are collections of scaffolds built from smaller subunits of assembled DNA sequence (Tuskan et al. 2006, Kelleher et al. 2007). A number of scaffolds are as of yet not positioned on the genome. The PopGenIE nomenclature does not use linkage groups, so all assembled units are referred to as scaffolds.

Table 4.2 presents an overview of the candidate genes tested, along with their characteristics such as size, location in the *P. trichocarpa* genome and function. Besides their assumed role

in vegetative dormancy control, several have a prominent function in flowering regulation, extensively studied in *Arabidopsis thaliana*. For that reason the names and accession numbers of the *Arabidopsis* homologues are mentioned as well. In Table 4.3, the methods for the primer design are explained for each of the gene regions tested, along with the primers tested. Lab names were assigned to the genes in order to show that it is definitely the *P. tremula* gene that is used in the analysis, rather than other *Populus* species. For instance, for PnFTL1 and PnFTL4 (*P. nigra*), the lab name was PtFTL1 and PtFTL4. For *P. trichocarpa* and *P. tomentosa* Carrière genes with prefix 'Pt' or 'PT', the names were kept.

Table 4.2 *P. tremula* candidate genes used in this study along with their characteristics

Arabidopsis locus	Full name	Arabidopsis accession	Populus locus	Populus gene size (bp)	Populus accession	Location in <i>P. trichocarpa</i> genome	Pathway	Function
/	ABSCISSIC ACID INSENSITIVE 1B	/	ABI1B	2490	AM690435	LG_VI:15011796-15012490	Phytohormone signalling pathway	Bud set and bud burst
TFL1	TERMINAL FLOWERING LOCUS 1 (Arabidopsis) / CENTRORADIALIS (Antirrhinum)	AT5G03840	PCENL1/ PopCEN	1126	AY383600.1	Scaffold_66:1231080-1231877	Long Day pathway (antagonistic to FT)	Inhibits flowering and bud burst
GA5	GIBBERELLIN 20-OXIDASE	AT4G25420	PtGA20ox8	1735	XM_002322396	LG_XV:9738552-9739354	Phytohormone signalling pathway	Promotes flowering and growth
CDKB	CYCLIN-DEPENDENT KINASE B	NM_115278.2	PtCDKB	2137	AY307372	LG_V:17784504-17785698	cell cycle progression	Cell activation and growth
CYCB	CYCLIN B	NM_001198208.1	PtCYCB	1200	FJ262735	LG_IX:293390-294677	cell cycle progression	Cell activation and growth
MFT	MOTHER OF FLOWERING LOCUS T	GQ177566	PnFTL4	2270	AB369068.1	LG_XV:3605860-3606770	Integrator of multiple pathways	Promotes flowering
FTL	FLOWERING LOCUS T LIKE	GQ176964	PnFTL1	814	AB183451	LG_XV:10159248-10160133	Integrator of multiple pathways	Promotes flowering
LFY	LEAFY	AT5G61850	PTLF	742	PBU93196	LG_XV:7932328-7932900	Induced by SOC1	Promotes flowering
SVP/ AGL24	SHORT VEGETATIVE PHASE / AGAMOUS-LIKE 24	AT2G22540	SVP/ AGL24	928	BU837680	LG_II:7637536-7638104	Repressor not clearly associated with a particular pathway	MADS transcription factor, inhibits flowering
SOC1/ AGL20	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 / AGAMOUS-LIKE 20	AT2G45660	SOC1/ MADS5	1084	XM_002302516	LG_II:11480765-11481314	Integrator of multiple pathways	MADS transcription factor, promotes flowering
SOC1/ AGL20	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 / AGAMOUS-LIKE 20	AT2G45660	SOC1/ MADS5	702	CA925124	LG_XIV:1774679-1775239	Integrator of multiple pathways	MADS transcription factor, promotes flowering
FVE	MULTICOPY SUPPRESSOR OF IRA1 4	AT2G19520	FVE	717	CA927540	Scaffold_145:528013-528537	Autonomous pathway	Transcription factor, promotes flowering

LG = Linkage Group

Table 4.3 The methods used for primer design for each *P. tremula* gene region tested and the primers tested. The expected fragment size is a result of in silico PCR performed using the PopGenIE database.

Populus locus	Populus accession	Method	Lab name	Forward primer 5' to 3'	Reverse primer 5' to 3'	Expected size (bp)
AB11B	AM690435 (Hall et al 2007)	Accession (<i>P. tremula</i>) BLAST in Genbank*, alignment with <i>P. balsamifera</i> sequences, primers chosen in conserved region	PtAB11B	AAAAGAGGACAGGGGAGGAA	AGTAATCGGTCGCCAATCAG	591
PCENL1/ PopCEN	AY383600.1 (Mohamed et al 2010)	Accession (<i>P. balsamifera</i> subsp. <i>trichocarpa</i>) BLAST in Genbank, alignment with other <i>P. balsamifera</i> subsp. <i>trichocarpa</i> and <i>P. nigra</i> sequences (Pn TFL), primers chosen in conserved region, not overlapping with similar PnFTL sequences	PtCENL1	CAGTCCAACAGGAAGCAGGT	TCCCTTGAAGCTGGAGTGGT	797
PtGA20ox8	XM_002322396 (Druart et al 2007)	Accession (<i>P. trichocarpa</i>) BLAST in Genbank and aligned with <i>P. nigra</i> , <i>P. tomentosa</i> , <i>P. alba</i> and <i>P. tremula</i> x <i>P. tremuloides</i> sequences, primers chosen in conserved regions	PtGA20ox	GCGGTCTTGGCTTTTCTTT	TTGAACAAAACCAATTGAACG	803
PtCDKB	AY307372 (Espinosa-Ruiz et al 2004)	Accession (<i>P. tremula</i> x <i>P. tremuloides</i>) BLAST in JGI** and Genbank, aligned with <i>P. trichocarpa</i> and <i>P. tomentosa</i> sequences, primers chosen in conserved regions	PtCDKB	TGGACACCGATCTCAAGAAA	CATTGTTGATGAACGCGACT	1195
PtCYCB	FJ262735 (Li et al 2009)	Accession (<i>P. tomentosa</i>) BLAST in Genbank and aligned with <i>P. trichocarpa</i> predicted protein and <i>P. trichocarpa</i> (from JGI) sequences, primers chosen in conserved regions	PtCYCB	AGCTCTGGCTGTGTCTCTCC	TAGGGCTGGTTCAGCTTTTG	1288
PnFTL4	AB369068.1 (Igasaki et al 2008)	Accession (<i>P. nigra</i>) BLAST in JGI and Genbank, aligned with <i>P. trichocarpa</i> MFT-like predicted protein sequences and primers chosen in conserved sequences	PtMFT	GCATGTTAGCAATGGGTGTG	TATGGAAGCAGGTGGGAGAC	911
PnFTL1	AB183451 (Igasaki et al 2008)	Primers as in Igasaki et al 2008 (for <i>P. nigra</i>). Tested with in silico PCR in Popgenie (<i>P. trichocarpa</i>)	PtFTL1	CGTTTTCGTTTCCTTAACCACTACAAA	GGAAAACCCACAACACTCTCTTAATCA	886
PTLF	PBU93196 (Rottmann et al 2000)	Accession (<i>P. trichocarpa</i>) BLAST in Genbank and aligned with other populus LEAFY-like sequences in <i>P. trichocarpa</i> , primers chosen in conserved regions	PTLF	CTTGCCAGCTTATAATTCCA	ATCTCCATCAAGGTCCACCA	573
SVP/ AGL24	BU837680 (Brunner & Nilsson 2004)	Accession (<i>P. tremuloides</i> x <i>P. tremula</i>) BLAST in Genbank and aligned with <i>P. trichocarpa</i> and <i>P. tomentosa</i> sequences. Primers chosen in LGII, +/- 200 bp each side of the overlapping sequence	PtSVP	TTTCTTGGGGTGTCTTCTGC	CTCCATGCCTCTATCTCTGTGA	569
SOC1/ MADS5	XM_002302516 (Tuskan et al 2006)	Accession (<i>P. trichocarpa</i>) BLAST in Genbank and aligned with <i>P. trichocarpa</i> , <i>P. tomentosa</i> and <i>P. tremuloides</i> sequences. Primers chosen in LGII, +/- 200 bp each side of the overlapping sequence	PtSOC1	TAGGGTGCAGGAACCATACC	CCTTTCATCTGGGTGGAGTT	550
SOC1/ MADS5	CA925124 (Brunner & Nilsson 2004)	Accession (EST <i>P. tremuloides</i>) BLAST in Genbank and aligned with <i>P. trichocarpa</i> , <i>P. tomentosa</i> and <i>P. tremuloides</i> sequences, primers chosen in LGXIV, +/- 200 bp each side of the overlapping sequence	PTM5	ACCCTAAGGTGCAAGAACCA	AAACCTTTTCTGCTGGGTGT	561
FVE	CA927540 (Brunner & Nilsson 2004)	Accession (EST <i>P. tremuloides</i>) BLAST in Genbank and aligned with <i>P. trichocarpa</i> sequences, primers chosen in scaffold_145, +/- 300 bp on each side of the overlapping sequence	PtFVE	GCCATATCATAAATGCTTTTCCA	CTTGATGGGGACTAGGACA	525

*Genbank: <http://www.ncbi.nlm.nih.gov/> **JGI: <http://www.phytozome.net/poplar>

EST = Expressed Sequence Tag, LG = Linkage Group, BLAST = Basic Local Alignment Tool

The candidate genes used in this study are discussed in more detail in this section, in particular their functions and previous studies. ABI1B and GA20ox are involved in hormonal pathways; and CYCB and CDKB are cell cycle regulators, while all others are involved in the floral regulatory pathway. In Figure 4.3, a simplified schematic shows the relationships between the flowering genes used, or related to genes used in this study. FT is the central gene in this pathway, with PtCENL1, PtMFT and PtFTL1 (used in this study) members of the same gene family. PtSOC1 and PTM5 are paralogues of each other, and homologues of SOC1. PTLF, PtFVE and PtSVP are the *P. tremula* homologues of respectively LFY, FVE and SVP.

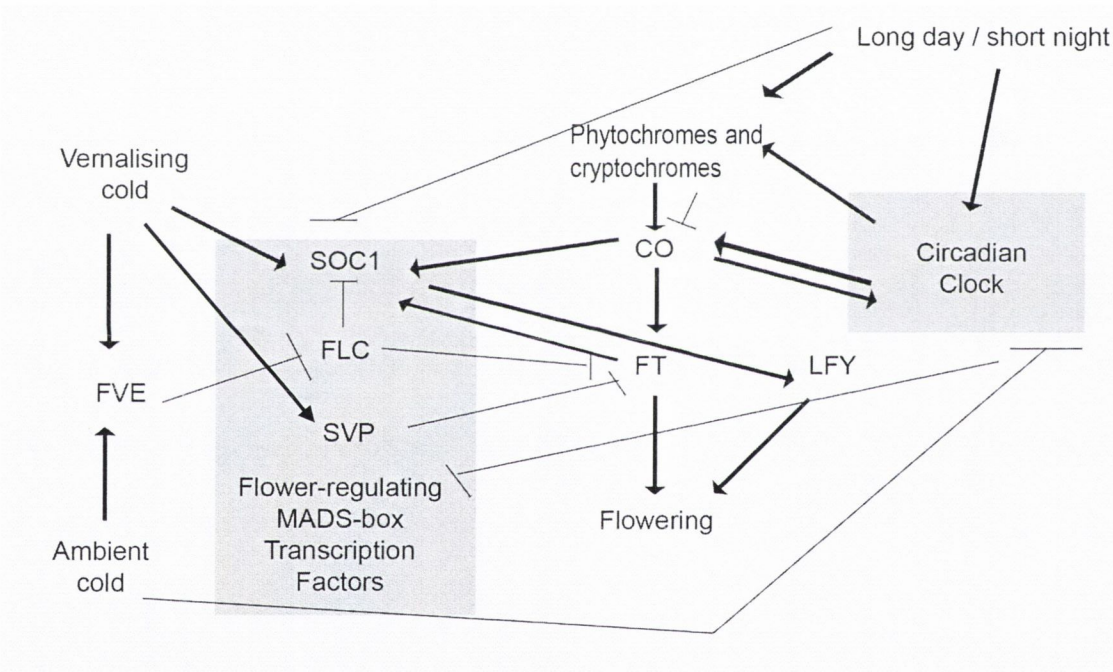


Figure 4.3 Simplified schematic, modified from Horvath (2009), showing part of the floral regulatory pathway, centred around flowering genes FLOWERING LOCUS T (FT) and LEAFY (LFY). CONSTANS (CO) is regulated through the perception of length of day and/or length of night by phytochromes and cryptochromes, and through the many components of the circadian clock. Subsequently, CO has a direct effect on FT. Cold temperatures (vernalisation) combined with long days result in altered expression of various flowering promoting or inhibiting transcription factors (proteins that control the transcription of certain parts of the DNA), such as SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), and SHORT VEGETATIVE PHASE (SVP). MULTICOPY SUPPRESSOR OF IRA 4 (FVE) is part of an autonomous pathway, indirectly having an effect on flowering through ambient temperature responses by chromatin remodelling. Only the most important genes, relevant to this study are mentioned, while many others are involved.

4.2.2.1 *ABI1B* (*ABA INSENSITIVE 1B*)

The *ABI1* gene family is responsible for reducing the plant's sensitivity to ABA (abscisic acid; Gosti et al. 1999). ABA is a plant hormone with functions in almost all plant organs: leaf abscission, seed dormancy, inhibition of cell division in cambial and leaf primordial tissues, root growth and stomatal control (Taiz and Zeiger 2006). ABA has also been indicated to be involved in cold hardiness as ABA insensitive or impaired *Arabidopsis* mutants were found to be less tolerant to freezing (Heino et al. 1990). The expression of PtABI1B (the *Populus* homologue of ABI1) was coincident with QTL affecting bud set and bud burst in hybrid poplars (*P. trichocarpa* x *P. deltoides*), and caused both phenological phases to occur earlier (Frewen et al. 2000). *Populus* ABI1B transcripts were detected at the time of bud set and increased during bud break in *P. trichocarpa* (A. Rohde, pers. comm. in: Chen et al. 2002). Garcia and Ingvarsson (2007) investigated the genetic diversity of PtABI1B in 4 *P. tremula* populations across Europe and discovered a relatively low nucleotide diversity and divergence. They did however find an excess of non-synonymous polymorphism in the coding region of this gene, which indicates a selective pressure maintaining the gene sequence (Garcia and Ingvarsson 2007).

4.2.2.2 *GA20ox* (*GA 20-OXIDASE*)

This gene is part of the hormonal pathway assembling gibberellic acid. Gibberellins (GA) have an antagonistic function to ABA, and exogenous treatment is well-known to break bud dormancy in woody plants, however only after a period of chilling (Paiva and Robitaille 1978). Members of the GA20ox gene family are upregulated by chilling in hybrid aspen (*P. tremula* x *P. tremuloides*; Rinne et al. 2011) and over-expression of the PttGA20ox1 gene induced elevated levels of bioactive GA1 (gibberellic acid 1), which stimulated growth, stem elongation and increased wood formation with longer xylem fibres in the same hybrid (Mauriat and Moritz 2009).

4.2.2.3 CYCB and CDKB (CYCLIN B and CYCLIN-DEPENDENT KINASE B)

CDKB and CYCB homologues are known cell division regulators and can be initiated independently of bud burst by local heating of the cambium, resulting in local reactivation of cambial growth in *P. sieboldiae* Miq. x *P. grandidentata* Michx. hybrids (Begum et al. 2007). Both genes show seasonal expression patterns in the cambium (Espinoza-Ruiz 2004, Druart 2007), but it is not clear whether temperature or daylength plays the main role: In a gene expression study using *P. tomentosa*, PtoCYCB and PtoCDKB transcript levels showed a positive correlation with mean daily air temperature in early spring (Li et al. 2009), while in a study using *P. tremula* x *P. tremuloides* hybrids, CDKB and CYCB were upregulated as a result of long day treatments (Karlberg et al. 2010).

4.2.2.4 CENL1 (CENTRORADIALIS LIKE 1)

CENL1 is a *Populus* orthologue of the *Antirrhinum* gene *CENTRORADIALIS* (*CEN*) and the *A. thaliana* gene *TFL1* (*TERMINAL FLOWER1*; Ruonala 2008). Members of the *CEN* gene family are involved in controlling shoot meristem identity and in delaying flowering in *A. thaliana* (Bradley et al. 1997). Transgenic experiments showed that *CENL1* has an effect on both flowering and vegetative dormancy control in poplar: suppression of *CENL1* induced flower formation, while over-expression caused delayed bud burst in *P. trichocarpa* (Mohammed et al. 2010). Real-time reverse transcription PCR (RT-PCR) revealed that *CENL1* transcription levels were increased prior to bud burst in vegetative buds of *P. trichocarpa* (Mohamed et al. 2010) and *P. tremula* x *P. tremuloides* (Rinne et al. 2011), while expression levels were low in inflorescence tissues (Mohamed 2006). Ruonala et al. (2008) also found expression of *CENL1* in the rib meristem (a meristematic region near the shoot apical meristem) of hybrid poplar, which had an effect on shoot elongation and transition to dormancy. The gene is under complex regulation of daylength, temperature and GA levels (Ruonala et al. 2008, Rinne et al. 2011).

4.2.2.5 *FT / TFL1* (FLOWERING LOCUS T / TERMINAL FLOWER 1) gene family: *FT1* (FLOWERING LOCUS T1), *MFT* (MOTHER OF FT AND TFL1), *FTL1* (FT/TFL1-LIKE1) and *FTL3* (FT/TFL1-LIKE3)

Members of the *FT/TFL1* gene family are all involved in instigating the onset of flowering. As shown in Figure 4.3, *FT* plays a central role in the flowering regulatory machinery. It is controlled by several transcription factors and indirectly by the phytochromes and cryptochromes (Figure 4.3). The relationships between the *FT/TFL1* family members were investigated in Lombardy poplar (*P. nigra*) and four distinct clades were discovered, even though all members showed a lot of similarities in their sequences and intron-exon organisation (Igasaki et al. 2008).

Although flowering genes are commonly associated with angiosperms, evidence from gymnosperms show that these genes also have further-reaching functions in perennial plants: *FT*-like genes expression patterns in Norway spruce (*Picea abies*) were correlated to growth rhythm responses under varying thermal and photoperiodic signals (Gyllenstrand et al. 2007). Indeed, Böhlenius et al. (2006) were the first to show that a poplar orthologue of *FT* (*PtFT1*) is a suppressor of short-day induced growth cessation and bud set, although the opposite effect was seen for the Norway spruce *FT*-like gene *PaFT4*. Lifschitz and Eshed (2006) even suggested that the primary target for *FT/TFL*-like genes is induction and termination of growth, and that induction of flowering could be seen as a pleiotropic effect, which occurs when one gene produces several phenotypic responses. Also expression of *FT* family member *MFT* (MOTHER OF FLOWERING LOCUS T) has been observed in flushing buds (Mohamed 2006).

4.2.2.6 *LFY* (LEAFY)

The *LEAFY Arabidopsis* gene has an important role in flowering promotion and floral meristem identity. *PTLF*, the *P. trichocarpa* *LEAFY* homologue, is mainly expressed in developing floral meristems in *Populus*, but was also clear in lateral vegetative meristems and young leaves

(Rottman et al. 2000). Although PTLF accelerated flowering when expressed in transgenic *Arabidopsis*, the only effects of overexpression in *Populus* were vegetative: a bushier habit and increased growth rate were observed but flowering was not affected. Moreover, the effects changed upon tree maturation. This suggests that PTLF is strongly subjected to regulation (Rottman et al. 2000).

4.2.2.7 SVP (*SHORT VEGETATIVE PHASE*), SOC1 (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*) and PTM5 (*P. tremuloides* MADS BOX 5)

SVP, SOC1 and PTM5 are type II MADS box transcription factors, closely related to each other. MADS-box transcription factors are conserved gene regions that have been shown to be important in the evolution and development of floral parts (Theissen et al. 2000). In a neighbour joining (NJ) tree composed by Leseberg et al. (2006) with poplar and *Arabidopsis* MADS-box sequences, SVP is part of a sister clade to PTM5 and SOC1, which actually are homologues of each other. SOC1 is named after *CONSTANS* (CO), a clock-regulated gene, which targets *FT* directly under influence of photoperception by PHYA (Phytochrome A; Figure 4.3). Besides timing of flowering, SOC1 was also found to affect determinacy of inflorescence and vegetative meristems (Melzer et al. 2008). Interestingly, the deactivation of SOC1 in the annual plant *Arabidopsis*, together with the suppression of another MADS box protein FUL (FRUITFULL), resulted in "perennial" features such as woodiness, recurrent growth cycles and vegetative buds (Melzer et al. 2008). PTM5 expression was found to be upregulated in spring and localised only in the vascular cambium and floral catkins (Cseke et al. 2003). SVP is a flowering repressor in *Arabidopsis* by negatively regulating FT. Even though it may have roles in several pathways, it has an important function in the response of plants to ambient temperature changes: loss-of-function *Arabidopsis* plants were found to be insensitive to temperature changes (Lee et al. 2007). In poplar, its function is not clear but Leseberg et al. (2006) found a very close relative of SVP in the MADS box gene family was expressed in the cambium tissue of *P. trichocarpa*.

4.2.2.8 FVE (*MULTICOPY SUPPRESSOR OF IRA 4*)

FVE is involved in flower and leaf development, and in one-dimensional cell growth in *Arabidopsis*. Historically the autonomous pathway (in which FVE regulates the flowering induction genes FT and LFY) was considered to act independently of the environment (i.e. independently of photoperiod changes). Ambient temperature effects were overlooked until it was found that *Arabidopsis* plants with mutations in FVE and other autonomous pathway genes flowered at the same time regardless of temperature changes, in contrast to wild type plants and those mutant in other pathways. FVE mediation is therefore required for ambient temperature responses of flowering in *Arabidopsis* (Blázquez et al. 2003).

4.2.3 Primer design

In order to design suitable primers for the PCR (polymerase chain reaction) of each gene region, a multiple alignment was made of the sequences resulting from the BLAST searches. In some cases *P. tremula* sequences were included, but *P. alba*, *P. balsamifera* L., *P. deltoidea*, *P. nigra*, *P. tomentosa* and *P. trichocarpa* sequences were also used. From the consensus sequence of these alignments, conserved regions of ± 20 bp were chosen for the primers using Primer3 software (Rozen and Skaletsky 2000). The primers were manufactured by Eurofins MWG Operon (Germany); and a test PCR was carried out using the extracted DNA of 5-7 randomly chosen samples. The main purpose for testing the primers was to find single bands for each sample in the electrophoresis of the PCR product, showing evidence that one single region in the genome had been amplified.

4.2.4 DNA extraction and PCR protocol

Of each individual, the soft tissue of 3-4 buds or 1cm² of dry leaf material was ground with a tissue grinder (QIAGEN TissueLyser II) to allow the extraction of total genomic DNA, using a DNeasy Plant Mini Kit (Qiagen GmbH) following the manufacturer's bench protocol.

PCR was undertaken on the total DNA in order to amplify the selected regions. A master mix was made up in a 1.5 ml Eppendorf tube, containing for each sample: (a) 22.2µl H₂O, (b) 3µl of 10x TBE buffer (Tris/Borate/EDTA; Sigma Aldrich; 15mM), (c) 0.9µl MgCl₂, (50mM), (d) 0.75µl of both forward and reverse primer (100µM), (e) 0.6µl dNTPs (10mM) and (f) 0.3µl (5 Units/µl) BIOTaq polymerase, for a 30µl reaction (all Bioline reagents). Subsequently, for each sample, 28.5µl of master mix was transferred into a micro tube (organised in plates or strips) and 1.5µl of total DNA was added. A negative control was included in each reaction.

The PCR process, using a Mastercycler Gradient PCR machine (Eppendorf) followed a standard "60°C protocol", with a 3min denaturation of the DNA at 94°C, followed by 30 cycles of amplification: (a) 1 min at 94°C, (b) 1min annealing at 60°C and (c) 1min extension at 72°C, and ending with a final extension of 10 minutes at 72°C. The subsequent samples were kept in a refrigerator at 4°C until gel electrophoresis could be carried out.

4.2.5 Gel electrophoresis

In order to verify the presence of total DNA after extraction and PCR product after amplification, all samples and controls were checked using gel electrophoresis. The gels were made of 1.5% agarose (Sigma-Aldrich) in 0.5x TBE buffer and heated in a microwave for 1 minute, after which SYBR Safe DNA gel stain (Invitrogen) was added (4µl per 100µl of agarose gel). The liquid gel was then poured into a holder with combs and left at room temperature to set for approximately 30 minutes. After setting, the agarose gel was placed into the electrophoresis gel rig (Multi Sub

Mini, Cleaver Scientific Ltd.) filled with enough 0.5x TBE buffer to cover the gel. The wells were loaded with a mix of 2µl of DNA loading buffer (Bioline) and 5µl of total DNA or PCR product of each sample, or negative PCR product. 5µl of ladder solution was also included in every gel run (Bioline EasyLadder I; 5 bands with size from 100 to 2000 bp). A voltage of 100V was applied to the gel for 25 minutes with a Consort EV265 power supply, after which the gel was viewed using a MiniBis Pro digital camera (DNR Bio-Imaging Systems). Positive bands were made visible by the reflection of blue light on the gel using an orange filter.

4.2.6 *DNA clean up and sequencing*

Positive, single banded PCR products of which the controls were negative were purified for sequencing using SureClean Plus solution (Bioline), in order to remove proteins, primers and dNTPs. After purification, following the manufacturer's protocol, the cleaned up PCR products were submitted to a new agarose gel electrophoresis (see section 2.5). From the photo of this gel, the DNA concentrations were determined with Gelquant v2.7.0, a programme from Bio-Imaging Systems. The programme calculates the mass of each of the bands of the purified PCR products by comparing with the bands in a well-separated EasyLadder I (50 ng of DNA per 5µl in each band). Knowing the mass per 5µl of PCR product, the necessary concentration could be made up for sequencing: for the gene regions larger than 1000 bp (PtCDKB, PtMFT) a concentration of 10ng/µl was made up in a total volume of 15µl, while for the other gene regions (between 500 and 1000bp) a concentration of 5ng/µl in 15µl was sufficient. The sequencing was outsourced to Eurofins MWG Operon, Germany. After sequencing results returned, a BLAST search was performed with the results in order to verify the correct genes were sequenced.

4.2.7 Sequence alignment and analysis

Alignment

The sequences of a particular region were assembled and aligned in contigs (a set of overlapping DNA segments) using Sequencher DNA Software 4.10.1 (Gene Codes Corporation), which also identifies polymorphic sites. When both forward and reverse sequences of a gene region of one particular individual were available, these were first aligned separately and subsequently checked for any inconsistencies in their sequences. All polymorphisms in the multiple sequence alignments were then manually verified using the chromatograms to exclude any scoring errors and the sequences were trimmed to the same size.

Nucleotide diversity

The software package used for the analysis of nucleotide diversity (π and S) was DnaSP 5.10.01 (Librado and Rozas 2009). Both π and S are measures of genetic variation, the raw material for selection and evolution. While π represents the frequency of mutant alleles (Nei, 1978), S (number of segregating sites) examines the number of polymorphic sites. Genetic diversity within and between the populations was calculated, including SNPs (single nucleotide polymorphisms), indels (insertion-deletions), and synonymous and non-synonymous mutations. The number of haplotypes H (sets of SNPs that are statistically associated) and the haplotypic diversity Hd (Nei 1987) were also calculated using DnaSP.

Neutrality tests

The nucleotide diversity patterns were tested for deviation from expectations under the neutral theory using DnaSP 5.10.01 (Librado and Rozas 2009). From a variety of neutrality tests, Tajima's D statistic (Tajima 1989) was selected for this study. Tajima's D test measures a standardized difference between two estimators of the expected amount of genetic variation ($\theta=4Ne\mu$, estimated by π and S , with N =population size and μ the mutation rate) under a neutral model. The neutral theory assumes that most mutations are either deleterious or neutral;

therefore the drivers of evolution are mutation and genetic drift rather than selective pressures. If evolution is neutral then π and S give the same value of θ (Page and Holmes 1998). Other indicators for neutrality (or rather for selective pressure) include for instance Ka/Ks or the ratio of non-synonymous to synonymous nucleotide substitution rates.

Differentiation

F_{st} or population differentiation index is a measure for diversity of populations, or how the genetic variation is distributed between the (sub-)populations (Weir and Cockerham 1984). It has a value between zero and one, with values closer to zero indicating more gene flow between populations, or between sub-populations. F_{st} was calculated as:

$$F_{st} = (H_t - H_s) / H_t$$

where H_t is the total heterozygosity (fraction of heterozygote alleles across all populations) and H_s is the heterozygosity of the populations (average of fraction of heterozygote alleles per population, Lowe et al. 2004). F_{st} was calculated for each SNP in all populations > 3 individuals and averaged for the whole sequence.

Allele frequencies

Allele frequencies were calculated for each SNP within populations of which 3 or more sequences of different individuals were available. The frequency of each allele (p and q) is the frequency of its homozygote form (f_{AA}, f_{aa}), plus half the frequency for all heterozygotes in which it is present (f_{Aa}):

$$p = f_{AA} + \frac{1}{2} f_{Aa} \text{ and } q = f_{aa} + \frac{1}{2} f_{Aa}$$

where $p + q = 1.0$ (Page and Holmes 1998). The frequencies p and q provide a simple description of the amount of genetic variation in a population.

Clinal variation in allele frequencies

Differences in the allele frequencies were used to test for clinal variation. R 2.12.2 and the *lm* package in R was used for linear regression and calculation of Pearson correlation coefficients in order to determine the importance of latitude, longitude and altitude on the genetic variation of the populations.

4.3 RESULTS

4.3.1 Gene regions and primers

After an extensive literature review a total of 14 gene regions were screened. Sequence alignments were prepared and primers developed. A panel of five to seven individuals was then used to test the primers. Seven of the 14 gene regions yielded successful sequences: PtABI1B, PtCDKB, PtFTL1, PTLF, PtMFT, PtSOC1 and PtSVP (Table 4.4, Appendix 4.2).

Table 4.4 Details of gene regions and primers in *P. tremula* with successful amplification and sequencing.

Lab name fragment	Populus gene size (bp)	Forward primer 5' to 3'	Reverse primer 5' to 3'	Expected fragment size (bp)	Obtained fragment size (bp)
PtABI1B	2490	AAAAGAGGACAGGGGAGGAA	AGTAATCGGTCGCCAATCAG	591	500-550
PtCDKB	2137	TGGACACCGATCTCAAGAAA	CATTGTTGATGAACGCGACT	1195	882*
PtFTL1	814	CGTTTTTCGTTTCCTTAACCACTACAAA	GGAAAACCCACAACACTCTCTTAATCA	886	246*
PTLF	742	CTTGCCAGCTTATAATTCCA	ATCTCCATCAAGGTCCACCA	573	398*
PtMFT	2270	GCATGTTAGCAATGGGTGTG	TATGGAAGCAGGTGGGAGAC	911	783*
PtSOC1	1084	TAGGGTGCAGGAACCATAACC	CCTTTCATCTGGGTGGAGTT	550	302*
PtSVP	928	TTTCTTGGGGTGTCTTCTGC	CTCCATGCCTCTATCTCTGTGA	569	427*

* size after trimming of sequences

Other gene regions tested were: PtCENL1, PtCYCB, PtFT1, PtFTL3, PtFVE, PtGA20ox and PTM5, but these resulted in poor quality sequences, unreadable sequences possibly due to the amplification of two or more paralogues, size variation, or a failure of either amplification or sequencing (Table 4.5). For PtGA20ox, double bands were visible in the gel after PCR, an indication of two or more regions being amplified. A gradient PCR with an array of annealing temperatures was carried out, in order to determine the optimal temperature for this particular region without amplification of other regions. From the gradient PCR the optimal temperature was established at 64°C. However further testing of the PtGA20ox region at this temperature showed no improvement.

Table 4.5 *P. tremula* loci tested but not selected for further analysis

Unsuccessful sequences	Reason	Samples tested for sequencing ^a	Quality of samples sequenced
PtCENL1	likely several paralogues sequenced	1.3, 5.1, 6.1, 6.2, 7.1*, 14.1, 18.2, 19.1, 26.1, 29.10	partially
PtCYCB	failed sequencing for most samples	5.1, 7.1* , 14.1, 18.2, 19.1, 26.1, 29.10	poor quality
PtFT1	no amplification in PCR or poor quality sequencing	5.10, 6.1, 6.2, 7.1*	partially
PtFTL3	no amplification in PCR	/	/
PtFVE	failed sequencing	1.3, 5.1, 6.1, 6.2, 7.1*, 14.1, 18.2, 19.1, 26.1, 29.10	/
PtGA20ox	double bands in gel	/	/
PTM5	likely several paralogues sequenced	4.9, 5.1, 5.9, 6.1, 6.2, 17.5, 18.2, 19.1, 38.1	ambiguous

^a Sample numbering: population number followed by sample number; sequences were obtained for samples in bold

* Population 7: *Populus alba*

The PtABI1B region was used for an initial trial but no polymorphism were observed in 500-550 bp of sequence from 5 individuals so this was not used further. Garcia and Ingvarsson (2007) previously found very low nucleotide diversity at the ABI1B locus in *P. tremula*, however they did discover two distinct haplotypes, which were not detected in this test. The six other gene regions did show some variation in the test sequencing and therefore more samples were selected for amplification and sequencing of these regions. Table 4.6 shows the number of individuals and populations used in the alignments for making up the consensus sequences and for further analysis, with the positions of each of the fragments within the *P. trichocarpa* genome. Between 40 and 64 individual sequences were obtained from 13-16 populations, giving a total of 324 individual fragment sequences. The fragments were located on 3 different scaffolds: scaffold 2 (PtSOC1 and PtSVP), scaffold 5 (CDKB) and scaffold 15 (PtFTL1, PTLF and PtMFT, with PtFTL1 and PTLF positioned relatively close to each other; Table 4.6). See Appendix 4.2 for the alignments of the consensus sequences with *P. trichocarpa* sequences. For PtSOC1 and PtSVP, using BLAST in the *P. trichocarpa* database resulted in several hits with varying overlapping lengths and similarities. In those cases, it was verified by the alignments that each of the sequences used to make up the consensus sequence was corresponding with the locations in the *P. trichocarpa* genome with highest similarities.

Table 4.6 Resulting region lengths of *P. tremula* genes, number of individuals and populations used in alignments, and the location of the consensus sequence in the *P. trichocarpa* genome.

Region	Length (bp)	Location in <i>P. trichocarpa</i> genome	Number of individuals	Number of populations
PtCDKB	882	scaffold 5: 25,354,568 - 25,353,688	54	15
PtFTL1	246	scaffold 15:13,358,948 - 13,358,712	64	14
PTLF	398	scaffold 15: 12,339,702 - 12,339,286	59	15
PtMFT	783	scaffold 15: 4,382,575 - 4,383,120	40	14
PtSOC1	302	scaffold 2: 11,439,795 - 11,440,023	63	16
PtSVP	427	scaffold 2: 7,663,023 - 7,663,456	44	13
Average	506.3333		54	14.5
Total	3038		324	87

Figure 4.4 illustrates the length of each region analysed and the relative proportions of introns and exons for each region, after alignment and trimming. PtCDKB was made up of a series of five relatively short intron- and exon sections (Roman numerals in Figure 4.4), while the other regions included one exon and one (PTLF), two (PtSOC1, PtSVP) or no introns (PtFTL1, PtMFT). The complete coding regions were sequenced for exons I-IV in PtCDKB and for the exons in PtSOC1 and PtSVP (Figure 4.4). Fragment lengths of the six gene regions ranged between 246 and 882 bp after alignment and trimming of the sequences (Table 4.6 and Figure 4.4), with a total of over 3 kb sequenced (Table 4.6). For PTLF, 20 base pairs were omitted from further analysis because the forward and reverse sequences did not fully overlap for many of the samples (Figure 4.4 and Appendix 4.2).

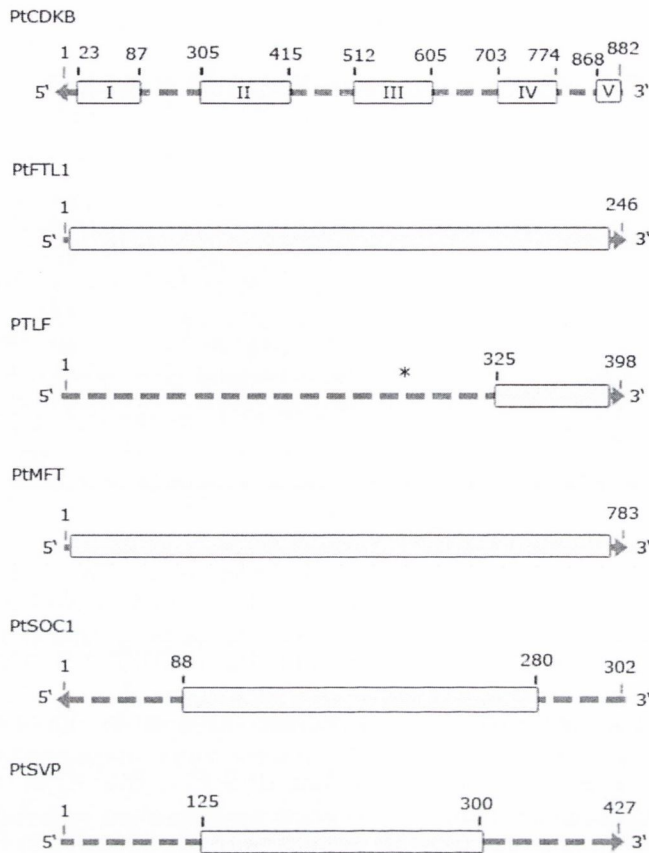


Figure 4.4 Schematic representation of the *P. tremula* gene regions selected for further analysis, showing the length of introns (non-coding, dashed line) and exons (coding, white bars). Numbers above the arrows are the number of base pairs, and the direction of the arrow the direction from the 5' to the 3' end of the DNA strand (direction as used in the analysis). Roman numerals are used to specify exons when >1 exons are present. In PTLF, 20 bp were omitted between bases 283-284, indicated by the asterisk.

4.3.2 Polymorphisms

The polymorphisms found in the 3kb sequenced belonging to six gene regions are summarised in Table 4.7. Three indels were detected in total: one of 8bp in PtSVP, and two in PtSOC1 (4bp and 2 bp long). The total number of SNPs found was 82 over all the gene fragments, with on average one SNP per 37 bp, which corresponds to 27 SNPs per kb. Of the 3kb sequenced, 1.8 kb were found in coding sequences and 1.2 kb were part of non-coding regions, in which most of the SNPs were found (52 non-coding SNPs out of 82 in total). Of the 30 SNPs in coding regions, 12 were synonymous (coding for the same amino acid) and 18 were non-synonymous. However, the non-synonymous mutations were only present in two of the six gene regions (Table 4.7).

Table 4.7 Summary of gene regions and polymorphisms detected in *P. tremula*. Indel sizes between parentheses.

Locus name	Region length analysed (bp)			Exons	Number of polymorphisms in region					
	Total	Coding	Non-coding		Indels	SNPs	Coding SNPs		Non-coding SNPs	bp/SNP
							syn	non-syn		
PtCDKB	882	354	528	5	0	18	2	0	16	49.0
PtFTL1	246	246	0	1	0	8	4	4	0	30.8
PTLF	398	73	325	1	0	19	0	0	19	20.9
PtMFT	783	783	0	1	0	18	4	14	0	43.5
PtSOC1	302	193	109	1	2 (4bp/2bp)	7	2	0	5	43.1
PtSVP	427	176	251	1	1 (8bp)	12	0	0	12	35.6
Total	3038	1825	1213	10	3	82	12	18	52	222.9
Average	506.3	304.2	202.2	1.7	0.3	13.7	2.0	3.0	8.7	37.2

4.3.3 Nucleotide diversity

All segregating sites (equal to the number of SNPs in the six sequences) were parsimony informative (Table 4.8). Nucleotide diversity gives a measure of the variation within a set of samples. It is the raw material on which selection can act. Estimates of total nucleotide diversity (π_{tot}) ranged from 0.00376 (for PtFTL1) to 0.0127 (for PTLF) between the six gene fragments, with an overall average of 0.00636 (Table 4.8). Non-coding sections were more variable than coding regions: the average π_{sil} was 0.0079 for silent sites (both non coding and synonymous), compared to coding nucleotide diversities π_{syn} and π_{ns} (for synonymous and non synonymous coding sites, respectively 0.0036 and 0.0019). This also shows that the level of diversity in coding sequences was higher for synonymous sites than for non-synonymous sites. High levels of non-synonymous nucleotide diversity were observed for PtFTL1 and PtMFT (π_{ns} : 0.0038 and 0.0075 respectively, compared to zero in the four other sequences). Between 10 (for PtFTL1) and 49 (for PtMFT) different haplotypes (H) were found in each of the gene regions, with haplotype diversity (Hd) ranging from 0.591 in PtFTL1 to 0.986 in PtMFT, and with an average of 0.804.

Table 4.8 Estimates of nucleotide diversity and neutrality estimates observed in *P. tremula*

Region	Length (bp)	S	Parsimony informative sites	Nucleotide Diversity				H	Hd	Tajima's D
				π_{tot}	π_{syn}	π_{ns}	π_{sil}			
PtCDKB	882	18	18	6	7.66	0	8.69	30	0.868	1.54 ns
PtFTL1	246	8	8	3.76	5.06	3.78	/	10	0.591	-0.87 ns
PTLF	398	19	19	12.66	0	0	10.43	31	0.929	1.18 ns
PtMFT	172	18	18	7.23	6.44	7.48	/	49	0.986	1.65 ns
PtSOC1	298	7	7	3.82	2.52	0	6.16	15	0.684	-0.27 ns
PtSVP	427	12	12	4.7	0	0	8.16	11	0.767	-0.42 ns
Average	403.83	13.67	13.67	6.36	3.61	1.88	8.36	24.33	0.80	

S: number of segregating sites; $\pi_{\text{tot}}/\pi_{\text{syn}}/\pi_{\text{ns}}/\pi_{\text{sil}}$: nucleotide diversity for total/synonymous/non-synonymous/silent sites (x 10⁻³); H: Number of haplotypes; Hd: Haplotype diversity; ns: not significant

4.3.4 Neutrality test

Based on Tajima's D neutrality test, there was no evidence found for departure from neutral evolution in any of the six gene regions. This result was expected for most gene regions, based on the synonymous mutations in the SNPs (see Table 4.7), except for PtFTL1 and PtMFT (with respectively 4 out of 8 non-synonymous SNPs and 14 out of 18). The Tajima's D estimates are listed in Table 4.8, all of which were non-significant on a 0.05 level. Positive values were obtained for the Tajima's D estimate of PtCDKB, PTLF and PtMFT, which can be generated by sampling across different paralogues (duplicated genes within a genome that take on a new function). This possibility was eliminated after BLAST searches in the *P. trichocarpa* genome. One paralogue for PtCDKB was found in scaffold 2, which was considerably shorter than the 882 bp of the PtCDKB consensus sequence; and for PTLF and PtMFT the search only yielded one copy of the sequence. A better method to rule out paraloguous genes would have been by gene cloning. However within the timeframe of this study it was not feasible to use this method.

4.3.5 Population differentiation

Overall genetic differentiation was determined for populations with 3–5 individuals, giving a mean *Fst* of 0.0461 over all polymorphic sites. The lowest *Fst* value per locus was found for PtSVP (-0.0857), which can be considered zero, as a negative differentiation is biologically meaningless. The highest differentiation of populations was located in the PtMFT region, with *Fst* value 0.1007 (Table 4.9).

Table 4.9 Heterozygosity (*Ht* and *Hs*) and differentiation (*Fst*) values, calculated over all *P.tremula* populations >3 and averaged over all SNPs

Region	Total individuals	Ht		Hs		Fst	
		Average	SD	Average	SD	Average	SD
PtCDKB	52	0.0876	0.1243	0.0856	0.1261	0.0727	0.0724
PtFTL1	64	0.0781	0.0579	0.0762	0.0619	0.0531	0.0599
PTLF	57	0.1856	0.1510	0.1800	0.1481	0.0387	0.0341
PtMFT	34	0.1544	0.0902	0.1527	0.0851	0.1007	0.1035
PtSOC1	61	0.1358	0.0516	0.1330	0.0509	0.0219	0.0558
PtSVP	38	0.0877	0.1296	0.0917	0.1281	-0.0857	0.2050
Total	306	Average*	0.1275	0.1213	0.1252	0.1198	0.0461

Ht: overall heterozygosity
Hs: heterozygosity in populations
Fst: population differentiation
SD: standard deviation
* over all SNPs

The low *Fst* value for PtSVP is due to negative values for all SNPs in this region except for SNP_419 (*Fst* 0.1133), which has a relatively high value. The variation in *Fst* values for SNPs and the actual distribution of the SNPs in the gene regions are represented in Figure 4.5, with all negative values depicted as zero. Note also in Table 4.9 that the standard deviations (SD) are large and this is likely due to the low number of samples used per population (3-5 individuals).

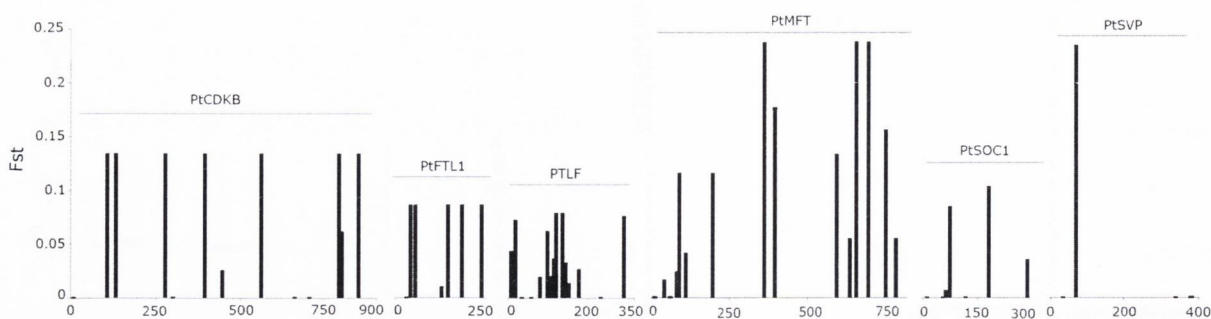


Figure 4.5 Distribution of SNPs in *P. tremula* sequences, with each SNP represented by an *Fst* value, showing the positions of SNPs and the variation of differentiation of individual SNPs within and between the gene regions.

4.3.6 Allele frequencies and clinal variation

Allele frequencies were calculated in each SNP for populations containing sequences of three individuals or more. All frequencies are tabled in Appendix 4.4. In order to find clinal trends with environmental factors in the genetic diversity, linear regressions were performed between allele frequencies at each polymorphic site and latitude and longitude. A majority of the Pearson correlation coefficients were close to zero, yet a number of SNPs were detected with correlation coefficients (r^2) higher than 0.25 (Figure 4.6), all of which were significant with p -values <0.05 . These correlations are only an indication that there could be clinal variation present in these SNPs, and can serve as a selection method for more detailed analysis.

The SNPs showing a possibility for variation in allele frequencies along latitude were PtCDKB SNP_296 ($r^2 = 0.28$, however with a p -value that is only significant at 0.1 level) and PtSOC1 SNP_186 ($r^2 = 0.64$, Figure 4.6). Along longitude, indications for variation were found in PTLF SNP_281 and SNP_318 ($r^2 = 0.51$ and 0.33 respectively) and in PtMFT SNP_778 ($r^2 = 0.62$, Figure 4.6).

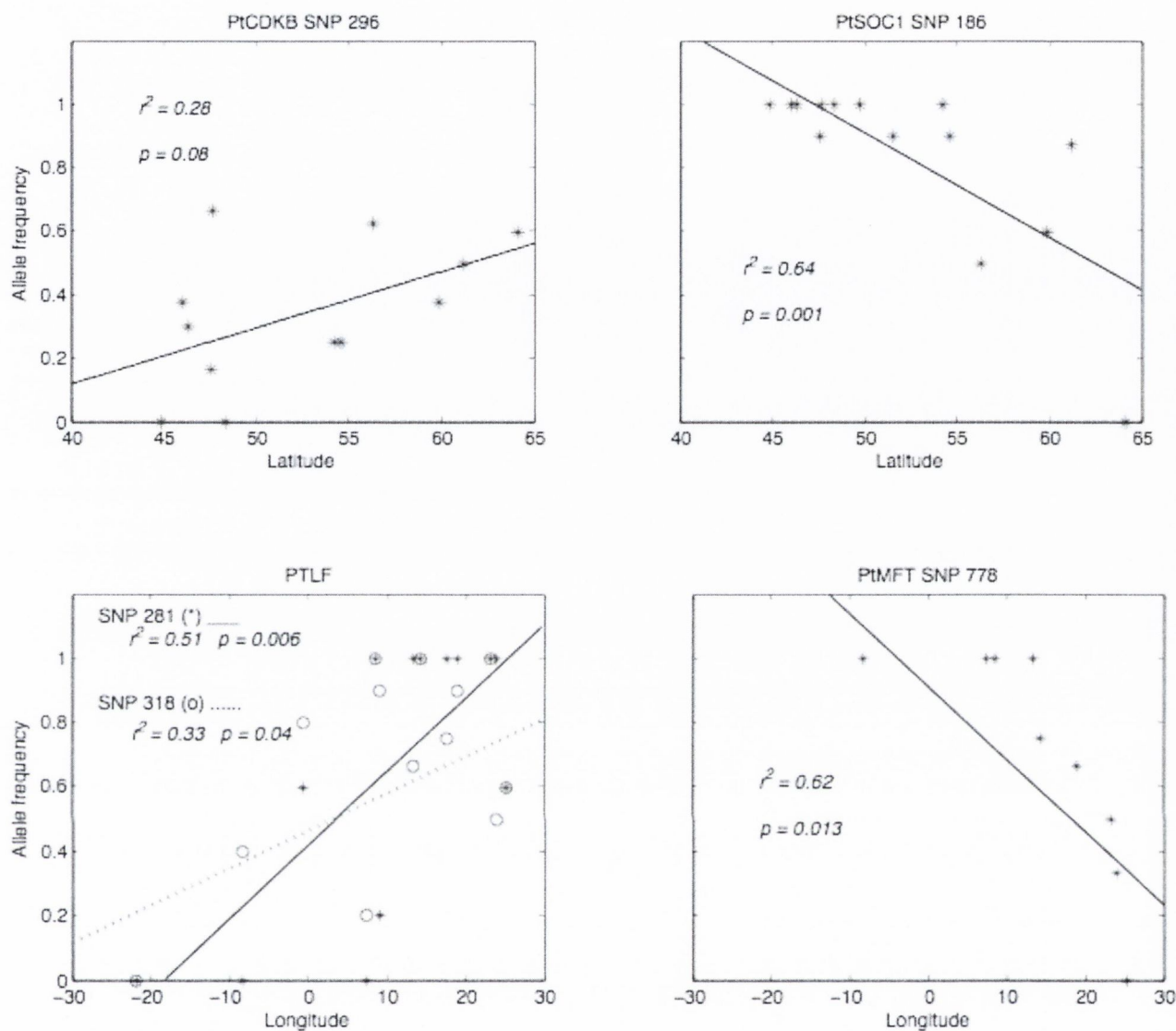


Figure 4.6 Allele frequencies of some selected SNPs vs. environmental gradients latitude (PtCDKB SNP_296 and PtSOC1 SNP_186) and longitude (PTLF SNP_281 and SNP_318, and PtMFT SNP_778). Correlations are significantly different from 0 ($p < 0.05$), except for the PtCDKB SNP ($p < 0.1$).

4.4 DISCUSSION

4.4.1 Nucleotide diversity and neutrality

In this first study of genetic diversity across such a broad range of European *P. tremula* populations the genetic basis of variation in bud burst and reactivation of growth in spring was explored by examination of nucleotide diversity in several candidate genes. Six candidate genes showing diversity were identified, putatively involved in temperature responses of spring phenological events in temperate trees. Whether the correct paralogue of a particular gene was sequenced, was verified by BLAST and by excluding PCR products showing double bands in the gel electrophoresis. However, a higher certainty would have been obtained by cloning.

The level of nucleotide diversity discovered across the six candidate genes (mean π_{tot} : 0.00636) lies within the previously reported range for poplar species. Ingvarsson (2005) found a higher diversity in five *P. tremula* genes sampled from four European populations (mean π_{tot} 0.0111), while the average π_{tot} of 0.0042 from the same populations, based on 77 gene fragments, was slightly lower (Ingvarsson 2008). In *P. trichocarpa*, the overall nucleotide diversity was measured at a relatively low 0.0018 (Gilchrist et al. 2006) and in *P. balsamifera* the π_{tot} observed was 0.0025 (Breen et al. 2009). Also in other tree species the reported values for dormancy-related genes range between similar values, e.g. 0.0062 and 0.0054 for *Quercus petraea* (sessile oak, Gailing et al. 2009, Derory et al. 2010) and 0.001 for *Pinus sylvestris* (Scots pine, Garcia-Gil et al. 2003). The large disparity (even between closely related species) shows that not only the systematic background is important but also the genes examined and the population history. Indeed, demographic events such as repeated population expansions and contractions during glacial and interglacial periods have been shown to influence the nucleotide diversity patterns we see today in European tree species (e.g. Kremer et al. 2002, Ingvarsson 2008).

Since none of the Tajima's D estimates were significant, neutrality could not be rejected for any of the gene regions in this study. This means that natural selection has had no significant influence on the variation observed and therefore, the variation that is present was caused by gene flow or random mutations. A negative estimate of Tajima's D was found for PtFTL1, PtSOC1 and PtSVP, indicating a large number of haplotypes and an excess of low frequency variants. These findings are not unusual: Ingvarsson (2005) and Ingvarsson (2008) also found mostly negative Tajima's D values for *P. tremula* and all of the nine bud burst candidate genes in oak tested by Derory et al. (2010) could not be considered deviating from neutrality. From the tree's perspective, it can be an advantage if the genes involved in dormancy traits are not put under strong selective pressures, particularly in the scope of climate change. Since climate change has a known effect on plant phenology, the neutrality of the genes and the high gene flow levels will allow the trees to respond relatively easily to changes in environmental conditions.

Although no deviation from neutrality was found, some SNPs show an indication for variation in their allele frequencies along a geographical cline. The detection of SNPs presents other possibilities as well. A recent study has for instance revealed that one single SNP in a non-coding region of the flowering gene *FLC* (FLOWERING LOCUS C) seems to be responsible for changes in flowering time in *Arabidopsis* (Caroline Dean, pers. comm.). With the rise of next generation sequencing technologies, large amounts of SNPs can easily be detected in model species (Geraldes et al. 2011). Thus, while the current study on a limited number of candidate genes revealed many SNPs, a larger throughput will find more, with more likelihood for detecting regions under selection.

4.4.2 Population differentiation

Adaptive population differentiation at the quantitative phenological traits has been proven for decades: e.g. in *P. trichocarpa*. (Dunlap and Stettler 1996), in *P. balsamifera* (Farmer and Reinholdt 1986) and also more recent, e.g. Vitasse et al. (2009) in several forest tree species. But

that does not correspond with the expectations for outcrossing species that genetic diversity is high within populations, compared to a rather low differentiation among populations (Gailing et al. 2009). Savolainen et al. (2007) and Hall et al. (2007) found evidence that the quantitative variation observed is not always reflected in the underlying genes: low *Fst* values were found at loci that resulted in strong genetic differentiation in common garden experiments.

In this study, the overall level of population differentiation *Fst* was established at 0.0461, with *Fst* values varying considerably between SNPs. However, this is merely an indication and no strong conclusions can be made from this number, as population sizes were very small (3-5 individuals). Furthermore, only the overall population differentiation was calculated here, as the small sample sizes per population made meaningful *Fst* calculations within populations impossible. The conclusions are therefore somewhat restricted, and can only be drawn based on differentiation per SNP or for the total group of populations, rather than on sub-groups (along environmental clines) or even on the populations themselves (pairwise *Fst*).

The calculated overall population differentiation was lower than, but close to 5%, the value above which differentiation is considered to be moderately present (Wright 1976). In other words, according to these calculations almost 5% of the genetic variation was due to variation among populations, while 95% was due to the variation within the populations. *Fst* values > 0.05 were found for gene regions PtCDKB, PtFTL1, PtMFT, and PtSVP, with highest values for SNPs in the PtMFT region (mean *Fst* 0.1, or 10% differentiation). According to Wright's guidelines (1976), these regions showed moderate genetic differentiation for the populations. The *Fst* were similar to those reported in previous research including four *P. tremula* populations from Sweden, France and Spain (Ingvarsson 2005): *Fst* ranged here between 0.04 and 0.161. Also in *P. balsamifera* relatively high *Fst* were found, with the highest value 0.256.

At least one negative *Fst* value was calculated in each gene fragment and included in the overall average. Here the average *Fst* value of PtSVP was also found to be negative. Even though

theoretically its value should be between zero and one, negative values are common in reality when there is more genetic variance within populations than between, and should be interpreted as zero (no differentiation or maximal gene flow, Weir 1996).

A certain amount of differentiation can be very valuable for plant populations, as it provides a possibility of intergroup selection: when evolutionary change is made possible on smaller groups it can happen more rapidly and more effectively. With rapid temperature increases due to climate change, this small amount of differentiation could be a determining factor for the survival of European aspen populations.

4.4.3 *Allele frequencies and clinal variation*

Allele frequencies deviated strongly in most cases from the Hardy-Weinberg equilibrium:

$$p^2 + 2pq + q^2 = 1$$

where p and q are the frequencies of the two respective alleles in a polymorphic site. Because neutrality could not be rejected from Tajima's D test, it is likely that the reason was the small population sample sizes in this study, rather than selection for a particular allele.

The basic regression method used for assessing clinal variation resulted in five SNPs showing indications for correlations with either latitudinal or longitudinal gradients. The *Fst* values of the five particular SNPs were low, contrary to an expected differentiation at a locus showing signs of clinal variation: 0 for PtCDKB SNP_296, 0.104 for PtSOC1 SNP_186, 0 and 0.08 for PTLF SNP_281 and SNP_318 respectively and at PtMFT SNP_778 *Fst* was 0.05. Although the *Fst* values were low, they were high relative to the other SNPs. Ideally, for the determination of clinal variation more individuals should be included per population, so that *Fst* values can be calculated within populations along the gradients. The functions of the gene regions in which the correlations were found (PtCDKB, PtMFT, PtSOC1 and PTLF) are quite varied, although all a role in the release of dormancy. PtCDKB is a cell cycle activator, which is also active outside

buds during spring growth, while the other three gene regions are active in flowering and vegetative buds. There was one transcription factor (PtSOC1), while PtMFT and PTLF are variants of the main flowering genes FT, FT and LFY. The implications for natural aspen populations, of a longitudinal cline in a SNP in one of these gene regions could be a different bud burst response to when environmental conditions become more similar due to climate change. Particularly the trees on the edges of the total distribution would not be able to adapt easily as recombination with individuals carrying the other allele is less likely. The trees occurring in the centre of the distribution have an advantage as they can adapt in either 'direction' of the allele. However, these implications assume that the location of the particular SNP showing the variation is vital, and that there are no other genes working simultaneously and balancing out any variations, which is not very likely, as bud burst and release of dormancy are complex traits involving many genes and pathways.

Local adaptations resulting in clinal variations can be caused by admixture (De Carvalho et al. 2010) or by selection along an ecological gradient (Endler 1977). Isolation by distance and genetic drift are not likely to play an important part as neutral markers generally have low differentiation among tree populations (Savolainen et al. 2007). With a longer growing season in southern Europe than in northern Europe, the decrease in growing season (and in average temperature) follows a latitudinal pattern, which many tree phenological traits are correlated with (Savolainen et al. 2007). The longitudinal correlations discovered in two genes in this study cannot be explained by temperature clines; however they are uncommon in phenological studies on forest trees: Chmura and Rozkowski (2002) found that longitude had the most influence on flushing and growth cessation date in beech provenance trials in Poland, in accordance with a pan-European study on beech (Von Wuehlisch et al. 1995): with increasing longitude, the trees showed lower heat requirements for bud burst to occur.

The correlation between phenological response and ecological gradients give evidence for phenotypic variation, but is there also genotypic evidence? Reports are mixed. Clinal variation

was found in growth cessation and in SNP frequency in *P. tremula* phytochromes (Ingvarsson et al. 2006); however no genetic variation was found along a cline in *Pinus sylvestris* phytochromes (Garcia-Gil et al. 2003). The most recent reports in *Populus* species and other forest trees have detected clinal variations in phenology on the molecular level and interesting possibilities for future research (Keller et al. 2011) however with low population subdivision (Alberto 2010, Ma et al. 2010). The candidate SNPs, potentially varying according to geographical clines, that are presented in this study need to be tested across a wider set of samples to determine if this is true clinal variation. Further gene expression studies would be necessary to determine the importance of the SNPs in gene variation and phenological responses.

It was beyond the scope of this study to verify the phenotypic variability in the natural populations sampled or the variability in phenology between the populations. A possibility to compare the genetic variability from this study with phenological variability is using satellite data as an estimate, for instance the map of growing season length shown in Figure 1.1.

4.4.4 Conclusions

In conclusion, high nucleotide diversity was found for six genes involved in dormancy control in *P. tremula*. In addition, low to moderate population differentiation was detected for these genes amongst natural *P. tremula* populations across Europe, although with this result it should be kept in mind that population sizes were small (3-5 samples per population). Both nucleotide diversity and population differentiation provide the raw material for adaptation of such populations to future climatic changes. Indications for clinal variation were detected for few genes across the geographical area examined. These genes deserve further investigations to assess their contribution to adaptive traits in natural populations.

The results suggest that geographic patterns are important for understanding the variation of European aspen, and illustrate the need for population genetic studies on genes related to phenology. A thorough knowledge of the genetic variation of natural populations can aid in

regulations for the sourcing of plants for forest management, breeding and conservation. *Populus* species are important worldwide for their use in the production of bio fuels, carbon sequestration and bio-remediation of toxins (Bradshaw et al. 2000). In 2011, the International Year of Forests, the United Nations want to strengthen sustainable forest management and conservation across the world in the light of climate change. Efforts to improve forests genetic resources have resulted in a total of 476 000 ha of forests being managed for *in situ* genetic conservation in 2010. However, 74% of this area is being used for only five species, mainly used in timber production (silver fir, Scots pine, Norway spruce, sessile oak and beech). Other species with more specific uses, such as *Populus* species, are often left out or are only scantily represented over their total geographical range. Therefore, the recent Forest Europe State of Europe's Forests Report (MCPFE 2011) states there is a need to develop species-specific genetic conservation strategies at pan-European level.

The results presented here show that the majority of diversity can be found on the scale of *P. tremula* natural populations, but certain alleles occur at different frequencies across the whole European range. Therefore, single scattered populations are not sufficient for a full representation of the genetic variants.

Although the focus of this study was on variation in genes involved in phenological responses to a particular environment, an important side note to make is that epigenetic effects more than likely play a large role in the tree dormancy cycle (Rohde and Junttila 2006, Yakovlev et al. 2006). However, it is still early days for this relatively new field, and epigenetic research techniques and the relevance for ecology and evolution of most organisms are still under discussion (Richards et al. 2010).

4.5 REFERENCES

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Chapter 5: General conclusions

5.1 INTRODUCTION

There is currently a high interest in climate change topics, not only within the scientific community, but also in popular media and from a policy point of view. Scientifically, climate change provides interesting challenges and opportunities, yet the many uncertainties about the effects on ecosystems imply an even greater challenge in finding practical solutions for conservation and adaptation, and in educating the general public (Primack and Miller-Rushing 2012). Therefore, the need for research in this topic remains, in order to contribute to finding better indicators for climate change, but also to estimate more accurately the impacts on the world, as we know it. A warming climate has the potential to affect many aspects of the life cycle of species and ecosystems, of which phenology, or the timing of seasonal biological events, is particularly sensitive (Parmesan and Yohe 2003, Walther 2003, Menzel et al. 2006). High interest in phenology studies was shown by the most recent IPCC Fourth Assessment Report (IPCC 2007), including a pan-European study reporting an advanced spring across Europe between 1971 and 2000 (Menzel et al. 2006) as an indicator of climate change.

The focus of this work was the timing of bud burst in two European species, *Betula pubescens* and *P. tremula*, and the implications for research on climate change impacts. The three-fold approach of this study, with (a) controlled environment experiments, (b) modelling techniques and (c) genetic research, resulted in a relatively broad analysis of different aspects of spring phenological events in trees, but with several interesting findings. New findings presented in the previous chapters included the determinism of chilling and photoperiod requirements in trees, showing for instance that chilling duration has a substantial effect on timing of bud burst, whereas photoperiod effect is weak. This chapter draws together the conclusions of the different chapters and places them into a wider context.

5.2 SUMMARY

The aim of this thesis was to provide a better understanding of some of the phenotypic and genetic mechanisms involved in bud burst of temperate trees by focusing on two tree species native to Europe. The first chapter expanded on the background of the topics of this work: trees, climate change, phenology, environmental and genetic control of phenology and the study species. Chapter 2 offered an experimental approach to investigate the timing of bud burst. The effects of chilling duration and photoperiod on the percentage and timing of bud burst in *P. tremula* and *B. pubescens* were quantified using cloned plants and controlled environment conditions. Results included a significant effect of chilling duration on the timing of bud burst of both species, however with a different response in speed and percentage of bud burst for both species. Photoperiod had a weaker effect on timing of bud burst for longer chilling durations, while shorter chilling durations had a strong effect on the percentage of bud burst. In the latter conditions, photoperiod was able to 'compensate' for a delay in dormancy release in *B. pubescens*, but not in *P. tremula*. Chapter 3 examined the parameters determining bud burst in the experiments described. The distribution factors of bud burst per tree revealed that not only the variation between the experimental conditions, but also the tree-to-tree variation and even the bud-to-bud variation within a single tree were relevant factors in understanding the experimental results. Finally, in Chapter 4, the genetic variation among natural populations of *P. tremula* was the focus of investigation. A set of candidate genes was identified, putatively involved in dormancy release and bud burst, and a set of single nucleotide polymorphisms within these genes was discovered. Nucleotide diversity was high and some indications were detected for (1) low population differentiation and (2) relationships between genetic variation in phenology-related genes and natural clines.

5.3 GENERAL DISCUSSION

The overall value of this work lies in its wide range of techniques, covering different aspects of phenology in trees. These different aspects are closely interlinked; for instance, the weak photoperiod effect on timing of bud burst is relevant for the selection of candidate genes in genetic studies, and the identification of the relevant genetic markers for bud burst is important for the modelling of large-scale impacts of climate change in forests and other ecosystems. Experimental data are needed for improvement of phenological models, and the comparison of models, in turn, could contribute to the discussion on how temperature and light interact for the timing of phenological events.

Environmental effects on tree phenology

A recent debate in the literature sparked a discussion on the relative importance of environmental cues (photoperiod vs. temperature) on tree phenology and the degree at which they will control phenology under a climate warming scenario. While Körner and Basler (2010) advocate the idea of a strong effect of photoperiodic limitation with spring warming, and therefore suggest that observed effects of temperature on spring phenological events cannot be extrapolated to future temperature conditions, others support the idea that spring phenology is highly dependent on temperature (Chuine et al. 2010). However, temperature responses are not straightforward, as warmer winters are likely to delay dormancy release, and warmer spring temperatures accelerate bud growth (Körner 2006, Morin et al. 2010). Evidence for both views show that the truth is neither black nor white, and differences exist between species. The focus of this study fits well into this debate and the results provided here showed a weak effect for photoperiod in the species *P. tremula* and *B. pubescens*, since long photoperiods in general did not compensate for a lack of chilling, reinforcing the view of Chuine et al. (2010). Therefore a delay in bud burst is a risk that should be taken into consideration when predicting the effects of climate change on ecosystems over the next century, and a net balance between spring advancement and delay working antagonistically should be assessed.

The results in this study indicate that *P. tremula* populations could face this delay in bud burst with climate change, resulting in a loss of competitive advantage in forests. Yet there was also a large nucleotide diversity found in phenology-related genes in European *P. tremula* populations, and high levels of gene flow indicating a good basis for adaptation to new climatic conditions. The chilling requirement or the thermal time requirement was higher for *P. tremula* than for *B. pubescens*, the latter reaching bud burst even under unfavourable conditions of short chilling duration and short photoperiod length. The risk for this species may lie in earlier bud burst with climate change, and therefore the possibility of being subjected to a late frost, which can have detrimental effects for trees (Bokhorst et al. 2009).

This work fits into previous research at Trinity College Dublin on tree phenology (Caffarra and Donnelly 2010, Caffarra et al. 2011a, 2011b). These studies included controlled environment experiments using several temperate tree species and modelling of the timing of bud burst creating a mechanistic model (Dormphot model). Our main finding of an increased timing and rate of bud burst with longer exposure to chilling corresponded with previous experimental results (Caffarra and Donnelly 2010). However, in the previous experiments a significant effect of photoperiod on bud burst was found for several species including *B. pubescens*, while photoperiod effects were found to be weak in this study. Further studies are needed to resolve the photoperiod-temperature issue, but several other factors could affect experimental results and should be taken into account. For instance, a recent report on environmental and genotypic control of dormancy in walnut trees found different processes to be at work between the endodormancy and ecodormancy stages (Charrier et al. 2011). Furthermore, a changing growth-promoting temperature, dependent on the state of dormancy has recently been reported in *B. pubescens* and *B. pendula* (Junttila and Hänninen 2012), while also the within-tree variation of timing of bud burst discussed in this study is a factor that should be included.

Research on genetic diversity of trees is vital for assessing and modelling climate change impacts on the distribution of species and the survival of woodlands, as survival is expected to increase with higher diversity within species and populations. Also phenotypic variation, fecundity, interspecific competition and biotic interactions play an important role (Aitken et al. 2008), yet models predicting species distributions are still mainly based on occurrence data (Garzón et al. 2011). Hence, there is a high need for genetic data, including the identification of genes under selection and within-species variability. The six candidate genes proposed have proven its effect on phenological traits as a response to temperature, based on literature and show high genetic diversity in this study. The results illustrate the need for population genetic studies on genes related to phenology, as the timing of life cycle events determines survival and fitness in trees, as shown in the experimental section of this study. Clearly, the field of genetic research is fast moving and large data outputs are possible in this field, particularly with the rise of next generation sequencing technologies and improved genome scanning techniques (Geraldes et al. 2011, Strasburg et al. 2012). However, more understanding is needed on the phenotypic and physiological functions of the loci under investigation (Strasburg et al. 2012), and on the role of epigenetic variation in phenotypic evolution (Richards et al. 2010).

With such a high number of factors to consider, the fate of forest trees under climate change remains uncertain. However, present and future knowledge could help determine which species and populations are at highest risk in natural ecosystems and how representative genotypes should be selected for forestry (Aitken et al. 2008). The results presented here show that the majority of diversity can be found on the scale of *P. tremula* natural populations, but certain alleles occur at different frequencies across the whole European range. Therefore, single scattered populations are not sufficient for a full representation of the genetic variants.

5.4 OVERALL CONCLUSIONS

Finally, returning to the hypotheses set in the aims of this study (section 1.7), the overall conclusions in this thesis were:

- (1) that a longer chilling period and a higher forcing temperature decreased the timing of bud burst and also increased the total percentage of bud burst in both species studied. However, the effect of photoperiod on timing and percentage of bud burst was minimal;
- (2) that despite the similar trends, *B. pubescens* and *P. tremula* showed a different response to the experimental conditions and varied considerably in chilling requirements and rate of bud burst;
- (3) that the distribution curves for bud burst provided new insights in the progress of bud burst on a tree-to-tree scale;
- (4) that the experimentally obtained data for bud burst under different conditions did provide a sufficient basis for fitting a basic mechanistic phenological model;
- (5) that significant genetic variation was present in dormancy-related traits between natural populations of an outcrossing species such as *P. tremula*;
- (6) and that genetic variation found among populations provided an indication of correlations with natural longitudinal and latitudinal clines.

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APPENDIX 4.1: List of all *Populus tremula* (*) samples collected

Population number	Material type	Number of samples	Country	Latitude ^a	Longitude ^b	Collector	Date collected
1	buds	11	Germany	47°38'	09°8'	Gregor Shmitz	06-Feb-09
2	buds	11	Germany	48°44'	9°05'	Zool. Bot. Garten Stuttgart	01-Feb-09
3	buds	1	Switzerland	47°22'	8°32'	Elisabeth Schneeberger	02-Feb-09
4	buds	11	Finland	62°39'	29°56'	Don Welsh	01-Feb-09
5	buds	10	Italy	45°59'	8°36'	Caretti Franco	06-Feb-09
6	buds	14	Latvia	56°19'	25°20'	Aija Liepkalne	03-Feb-09
7*	buds	10	Belgium	50°51'	4°41'	Paul Uytendaele	29-Jan-09
8	buds	10	Latvia	57°21'	26°46'	Guna Usele	01-Feb-09
9	buds	10	Latvia	56°40'	22°0'	Līga Strazdina	01-Feb-09
10	buds	12	Finland	61°01'	25°25'	Ritva Hiltunen	01-Feb-02
11	buds	12	Czech Rep.	49°44'	13°22'	Vacvlava Peskova	Feb-09
13	buds	5	Moldova	48°09'	27°17'	Vasile Spirin	Feb-09
14	buds	10	Lithuania	54°37'	23°17'	Arnas Balsevicius	Feb-09
16	buds	7	Germany	49°47'	7°57'	Berthold Meyer	Feb-09
17	buds	10	Sweden	59°51'	17°38'	Bot. Gard. Uppsala Univ.	Feb-09
18	buds	12	Finland	61°12'	24°00'	Jaakko Saarinen	01-Mar-09
19	buds	16	France	44°50'	00°33' W	Laurent Lestourmeaud	01-Mar-09
20	buds	10	Ukraine	51°28'	31°18'	Mykyta Peregryn	15-Feb-09
21	buds	11	Czech Rep.	50°25'	14°34'	Miloslav Studnička	22-Feb-09
22	buds	10	Finland	67°1'	24°40'	Matti Yli-Rekola	02-Mar-09
23	buds	12	Switzerland	46°17'	7°29'	Pascal Vittoz	14-Feb-09
24	buds	9	Slovakia	48°29'	18°26'	Ivan Borin	22-Feb-09
25	buds	17	Italy	37°45'	15°00'	Rosario Galesi	01-Mar-09
28	buds	10	Germany	52°13'	13°51'	Hans Sonnenberg	05-Mar-09
29	buds	10	Austria	48°18'	14°17'	Ferdinand Lenglachner	05-Mar-09
30	buds	5	Hungary	47°31'	18°56'	Attila Makay	12-Mar-09
31	buds	10	Norway	66°08'	13°24'	Stig Lundmo	01-Apr-09
33	buds	7	Austria	48°02'	15°00'	Franz Tod	23-Mar-09
34	buds	10	Lithuania	55°50'	23°22'	R. Vainoziene	14-Mar-09
37	leaves	10	Germany	53°06'	8°14'	Dr Detlev Metzger	02-Apr-09
38	leaves	6	Ireland	54°14'	8°23' W	Annelies Pletsers	05-Jul-09
39	leaves	10	Ireland	51°15'	9°55' W	Colin Kelleher	23-Aug-09
40	leaves	4	Ireland	52°01'	10°48' W	Colin Kelleher	Sep-09
41	leaves	10	Iceland	65° 51'	17°50' W	Heidi Salstad	Jul-09
42	buds	12	Poland	50°04'	20°25'	Dr Leszek Bartkiewicz	14-Mar-10
43	leaves	10	France	48°36'	01°57'	Didier Chagot	12-May-10
44	buds	17	UK	50°48'	04°06' W	Robert Wolton	19-Apr-10
45	leaves	10	Scotland	various	various	John Parrott	17-May-10
46	buds	8	Belgium	various	various	Kristien Vandermijnsbrugge	09-Apr-10
47	leaves	5	Spain	42°00'	02°51' W	Colin Kelleher	Aug-10
48	leaves	13	Norway	63°21'	10°35'	Annelies Pletsers	09-Sep-10

^aDegrees North

^bDegrees East, with exception of populations 19, 38, 39, 40, 41, 44 and 47 (Degrees West)

**P. alba* in population 7

APPENDIX 4.2: Samples with successful sequences used in analysis

Population number	Gene region						
	PtCDKB	PtFTL1	PTLF	PtMFT	PtSOC1	PtSVP	
	Samples used						
1	1.3	1.3	1.3		1.3	1.3	
	1.6	1.5	1.5		1.6	1.5	
	1.8	1.6	1.6		1.7	1.8	
4			4.9	4.9	4.9		
	5.7	5.1	5.7	5.1	5.1	5.1	
	5.8	5.7	5.8	5.7	5.7	5.7	
5	5.9	5.8	5.9	5.8	5.8	5.8	
	5.10	5.9	5.10	5.9	5.9	5.10	
		5.10			5.10		
6	6.1	6.1	6.1	6.1	6.1	6.1	
	6.2	6.2	6.4	6.2	6.2	6.2	
	6.4	6.3	6.5	6.3	6.3	6.3	
	6.5	6.4		6.4	6.4	6.4	
		6.5		6.5	6.5	6.5	
	11.1	11.1	11.1	11.1	11.1	11.1	
11	11.4	11.2	11.2	11.2	11.2	11.2	
		11.3	11.3	11.3	11.3	11.3	
		11.4	11.4	11.4	11.4	11.4	
		11.5	11.5		11.5	11.5	
	14.1	14.1	14.1	14.6	14.1	14.1	
	14.7	14.6	14.6	14.7	14.6	14.6	
14	14.8	14.7	14.7	14.8	14.7	14.7	
	14.9	14.8	14.8	14.9	14.8	14.8	
		14.9	14.9		14.9	14.9	
17	17.6	17.6	17.6	17.6	17.6	17.6	
	17.8	17.8	17.7	17.7	17.7	17.7	
	17.9	17.9	17.8		17.8	17.10	
	17.10	17.10	17.9		17.9		
			17.10		17.10		
	18.2	18.2	18.2	18.2	18.2	18.2	
18	18.4	18.3	18.3	18.3	18.3	18.3	
	18.6	18.4	18.4	18.4	18.4	18.4	
		18.5	18.5		18.6	18.5	
		18.6	18.6				
	19.1	19.1	19.1	19.1	19.1	19.1	
	19.2	19.2	19.2	19.5	19.3	19.2	
19	19.3	19.3	19.3		19.4	19.3	
	19.4	19.4	19.4		19.5		
	19.5	19.5	19.5				
20	20.3	20.1	20.1	20.1	20.1		
	20.5	20.2	20.2		20.2		
		20.3			20.3		
		20.4			20.4		
		20.5			20.5		
	23.1	23.1	23.1	23.1	23.1	23.3	
23	23.2	23.2	23.2	23.3	23.2	23.5	
	23.3	23.3	23.3	23.5	23.3		
	23.5	23.5	23.5		23.5		
	23.6	23.6	23.6				
	26.1			26.1			
26							
29	29.3	29.1	29.3	29.1	29.4	29.6	
	29.4	29.3	29.4	29.3	29.6	29.10	
	29.6	29.4	29.9	29.4	29.9		
	29.9	29.6	29.10	29.9			
	29.10	29.9					
	30.2	30.1	30.1	30.2	30.1	30.2	
30	30.3	30.2	30.3	30.3	30.2	30.3	
	30.4	30.3	30.4	30.4	30.3	30.4	
	30.5	30.4			30.4		
		30.5			30.5		
	38.1	38.1	38.1	38.1	38.1	38.1	
	38.2	38.2	38.2	38.2	38.2	38.2	
38	38.3	38.4	38.3	38.3	38.4	38.3	
	38.5	38.5	38.4		38.5		
			38.5				
41	41.1	41.1	41.1		41.1	41.1	
	41.2	41.2	41.2		41.2	41.3	
	41.3	41.3	41.3		41.3		
	41.4		41.4				
	41.5		41.5				
Number of samples per region	50	64	60	40	62	44	Total: 320

APPENDIX 4.3: Position of gene regions (consensus sequence) in *Populus trichocarpa* genome

1.PtABI1B

Database: Ptrichocarpa_v156_assembly_masked; 2,518 sequences; 417,137,944 total letters
scaffold_6

Length = 26,894,541; Score = 482.205 bits (243), Expect = 1.34173e-134 Identities = 292/309 (94%), Gaps = 5/309 (1%), Frame = +1 / -1

```
Query: 13      ATTGTTGACAGATGCCATGGCTTAATTAGAGAAG---AAACAGAAAAAATCAGGGTAG 68
              |||  |||
Sbjct: 22778983 ATTGCTGACAGATGCCATGGCTTAATTAGAGAAGGAAGAAACAGAAAAAATCAGGGTAG 22778924

Query: 69      AAAAACATACCAATAGACCTTGACATTGCTAGAACACCAAAGACACGATGCCCATTCCAC 128
              |||  |||
Sbjct: 22778923 AAAAACATACCAATAGACCTTGACATTGCTAGAACACCAAAGACACGATGCCCATTCCAC 22778864

Query: 129     TGTATGACCTTGCCTCCAGCTGCTTCTATCCTTGCATACTCATCTTCTCGGTTTGGCTAA 188
              |||  |||
Sbjct: 22778863 TGTATGACCTTGCCTCCAGCTGCTTCTATCCTTGCATACTCATCTTCTCGGTTTGGCTAA 22778804

Query: 189     GATTAGATTTAGCAAGAGAATCAAATGAACAAGAAACAGCTTTCCTGTCTATTACTACCA 248
              |||  |||
Sbjct: 22778803 GATAAGATTTAACACGAGAATCAAATGAACAAGAAACAGCTTTCCTGTCTATTACTACCA 22778744

Query: 249     TTAAAACTTAAATATCCAAGCTTACAGTTGTAAAAGGCTCTTACTTTATGA-CCACTGA 307
              |||  |||
Sbjct: 22778743 TTAAAACTTAAATATCCAAGCTTACAGTTGTAAAAGGCTCTTACTTTATGATCCACTGA 22778684

Query: 308     TAATGCCAT 316
              |||
Sbjct: 22778683 TAATGCCAT 22778675
```

2. PtCENL-1

Database: Ptrichocarpa_v156_assembly_masked; 2,518 sequences; 417,137,944 total letters
scaffold_4

Length = 23,188,140, Score = 858.563 bits (422), Expect = 0

Identities = 549/602 (91%), Gaps = 19/602 (3%), Frame = +1 / -1

Query: 3 TAGATCAGGGTGAAGAATCATACCAAATGTGGCATCTGTTGTCCTGGGATGTCAGTTAC 62
|||||
Sbjct: 20552965 TAGATCAGGGTGAAGAATCATACCAAATGTGGCATCTGTTGTCCTGGGATGTCAGTTAC 20552906

Query: 63 TATCCTGTAATGATATTGGTCAAATCAAGGTAAAGCAATGAGGATGTGTTTTAACTGTA 122
|||||
Sbjct: 20552905 TATCCTGTAATGATATTGGTCAAATCAAGGTAAAGCAGTGAGGATGTGATTTTAACTGTA 20552846

Query: 123 CYTTTCTTACTATTTTTATTACTCAACATGATTACACCTTTTCTACTTGAGTTGTGTGR 182
| |||||
Sbjct: 20552845 CTTTCTTACTATTTTTATTATTCTACATGATTACACCTTTTCTACTTGAGTTGTGTGA 20552786

Query: 183 GAAATCTGGAAATGATAGAGGCAGCTTTTTAACTATTTTTGGGTGTTACTTGGAATTC 242
|||||
Sbjct: 20552785 GAAATCTGGAAATGATAGAGGCAGCTTTTTAACTATTTTTGGGTGTTACTTGGAATTC 20552726

Query: 243 AATTAAYAMTTGKT--CAMTTATCATTCATGGAAATAATTCCTTGCTAGCTTGAGTTMT 300
||||| | ||| | || ||||| ||||| ||| |||||
Sbjct: 20552725 AATTAATACTTGTTTACAGTTATCATTAAATGGAAATAATTCCTTGCTAGCTTGAGTTCT 20552666

Query: 301 WYWCATATAGGWGRAGAGATTATTTAGAGAAATTGATATACATACCAAGTGAGGTGCTCC 360
||||| | ||||| ||||| |||||
Sbjct: 20552665 ATACATATAGGTGAAGAGATTATTTAGAGAAATTGAT----ATACCAAGTGAGGTGCTCC 20552610

Query: 361 CTGAGGTATGGATCACTAGGACCAGGAACATCAGGGTCTGTCATGACCTGCAGCCATCAA 420
|||||
Sbjct: 20552609 CTGAGGTATGGATCACTAGGACCAGGAACATCAGGGTCTGTCATGACCTGCAGCCATCAA 20552550

Query: 421 GGTATMCAGTGAGAACASTATCAAAGAAGAGAAAAGGASMTCTYGGGCATGGAAGAA 480
||||| ||||| ||||| | ||| || || |||||
Sbjct: 20552549 GGTATACAGTGAGAACACATCAAAGAAG--GAAAAAGAGCTCTTGGGCATGGAAGAA 20552492

Query: 481 AAAACACAAACATGTATCATACCAAGGTGAAAAAGGATCTCATATCACCTCCATGAACCT 540
||| |||||
Sbjct: 20552491 CAAA-----TATCATACCAAGGTGAAAAAGGATCTCATATCACCTCCATGAACCT 20552442

Query: 541 CAACTTTAGGTTTTTATGAGTTACTGCAGATGGRWRSTAGCTCATGGCCATTAAAAACCTGC 600
||||| ||||| |||||
Sbjct: 20552441 CAACTTTAGGTTTATGAGTTACCGCAGATGGGAA-TAGCTCATGGCCATTAAAAACCTGC 20552383

Query: 601 TT 602
||
Sbjct: 20552382 TT 20552381

3. PtCDKB

Database: Ptrichocarpa_v156_assembly_masked; 2,518 sequences; 417,137,944 total letters
scaffold_5

Length = 25,802,683, Score = 1449.28 bits (722), Expect = 0
Identities = 836/883 (94%), Gaps = 3/883 (0%), Frame = +1 / -

Query: 1 TTTGGAGTACCTAATAACCTGTACAAGAGCTTCAAAAYGTATATTAAAGAGAAGCCTAAT 60
|||||
Sbjct: 25354568 TTTGGAGTACCTAATAACCTGTACAAGAGCTTCAAAACGTATATTAAAGAGAAGCCTCAT 25354509

Query: 61 TGACAAAGCAAGATCAGAACCATATCCATCKITATGMAAAATCATTGACCTGAAAATAT 120
|||||
Sbjct: 25354508 TGACAAAGCAAGATCAAAACCATATCTATCGTTATGCAAAATCATTGACCTGAAAATAT 25354449

Query: 121 GCAGGAGCTGTTGCAGTTCAGAGTCTCCAGGGAAGAGTGCTTGCTTTGTGGCCAGTTCAG 180
|||||
Sbjct: 25354448 GCAGGAGCTGTTGCAGTTCAGAGTCTCCAGGGAATAGTGCTTGCTTTGTGGCCAGTTCAG 25354389

Query: 181 CTASAATTTAGGCAAAACACAAACACAACAATTAAGCAATGCTAGAGYTGCGAGATTAGT 240
|||||
Sbjct: 25354388 CTAGAGTTTAGGCAAAACACACACAACAATTAAGCAATGCTAGAGTTGGCAGATTAGT 25354329

Query: 241 TTAAGAGATGAAGCGATAAGAATAAGGAAATCCATACCAATATACAGCCAACAGACCAT 300
|||||
Sbjct: 25354328 TTAAGAGATGAAGCGATAAGAATAAGGAAATCCATACCAATATACAGCCAACAGACCAT 25354269

Query: 301 ACATCCACTGCAGTTGAGTAATGGGTWGGCCCCAAAAGGACTTCAGGAGATCTATACCAC 360
|||||
Sbjct: 25354268 ACATCCACTGCAGTTGAGTAACGGGTAGCCCCAAAAGGACTTCAGGAGATCTATACCAC 25354209

Query: 361 AAAGTCAATATCTGAAACAGAAATCCACTCAATTAGCTCAACAACTGATATTGTAAGA 420
|||||
Sbjct: 25354208 AAAGTCAATATCTGAAACAGAAATCCACTCAATTAGCTCAACAACTGATATTGTAAGA 25354149

Query: 421 GCATGACAAAAAGATGGTARGTAGCAAGCATGTATTAATTTAATTACCTCATGGGTATAC 480
|||||
Sbjct: 25354148 GCATGATAAAAAGACGGTAAGTAGCAAGCGTGTATTAATTTAATTACCTCATGGGTATAC 25354089

Query: 481 TTCTTAATAGGMAGAGTAAATGCTCGAGCTAGTCCGAGATCAGCTATTTTAAGCATCATA 540
|||||
Sbjct: 25354088 TTCTTAATAGGAAGAGTAAATGCTCGAGCCAGTCCGAGATCAGCTATTTTAAGCATCGTA 25354029

Query: 541 GTCTTGCGGTCCATCAAGAGATTATGAGGCTTAAGATCCCTGAGAGSAAGCGAGATAAAG 600
|||||
Sbjct: 25354028 GTCTTGCGGTCCATCAAGAGATTATGAGGCTTAAGATCCCTGAGAGGAAGCGAGATAAAG 25353969

5. PTLF

Database: Ptrichocarpa_v156_assembly_masked; 2,518 sequences; 417,137,944 total letters
scaffold_15

Length = 15,134,944, Score = 626.653 bits (308), Expect = 3.61106e-178

Identities = 379/417 (90%), Gaps = 20/417 (4%), Frame = +1 / -1

```
Query: 2      GCCACTGCCTCCCATGTCCCTCTTCCACCGCTCCCTGCTGCCTCCTTGTCTTGCTGTACT 61
             |||
Sbjct: 12339702 GCCACTGCCTCCCATGTCCCTCTTCCACCGCTCCCTGCTGCCTCCTTGTCTTGCTGTACT 12339643

Query: 62      GGCTCCTCAGAGAAACCTGARCACWCAAYCACCCAAGAAACAAGCAAAAATAAAA----- 116
             |||
Sbjct: 12339642 GGCTCCTCAGAGAAACCTGAACACACACACCACCCAAGAAACAAGCAAAAATAAAAATTA 12339583

Query: 117     -----GTYTAATAAGTTCAITGTTATTTAATWTTTCGAAACAATCAGATTT 161
             ||
Sbjct: 12339582 AGGAAAGGAAAATAAGTTTAATAAGTTCATGTTATTTAATTTTCGAAACAATCAGATTT 12339523

Query: 162      TGTTCAITTTATTTTCTAGAAACCAGCATGAGCTCAAAGCTATTTAASCTAACTATTACCTT 221
             |||
Sbjct: 12339522 TGTTCAITTTATTTTCTAGAAACCAGCATGAGCTCAAAGCTATTTAAGCTAACTATTACCTT 12339463

Query: 222      GAGAACTGATTTTAAGCTAAGYATGTATGGCAATTAACAAGAAACCAAAGCTAGCWRACC 281
             |||
Sbjct: 12339462 GAGAACTAATTTTAAGCTAAGTATGTATGGCAATTAACAAGAAACCAAAGCTAGCTAACC 12339403

Query: 282      TGRACCTAGTTTARGAGAATTACTACTCTAATATTGAKCATGGTGATTAATTAGCAAGAAA 341
             |
Sbjct: 12339402 TTGACTTAGTTAAGAGAATTACTACTCTAATATTGAGCATGGTGATTAATTAGCAAGAAA 12339343

Query: 342      MCAACCCTGCAAAGTAGAATTGATATGWCTAGAGTAAACATACAAATGGTTTACAAGC 398
             |||
Sbjct: 12339342 CCAACCCTGCAAAGTAGAATTGATATGACTAGAGTAAACATACATTGGTGTACAAGC 12339286
```

Database: Ptrichocarpa_v156_assembly_masked; 2,518 sequences; 417,137,944 total letters

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Query: 1          GACGAGCTTTACACTCTGGTTAGATTTTAGCTGC TTATGTGTTTTTACTTTTTATGT 60
                |||
Sbjct: 4382575    GACGAGCTGTACACTCTGGTTAGATTTTAGCTGCGTATGTGTTTTT-----ATGT 4382626

Query: 61         TCCTGCATGCAGGTGTATTTTATGCTAAAAATAATATGTACTGTGTTGTTTTCTCTTTGT 120
                |||
Sbjct: 4382627    TCCTGCATGCAGGTGCTCTTTTATGCTAAAAATAATATGTACTGTGTTATTTTCTCTTTGT 4382686

Query: 121        TTGTGCATGTCGATCTGCTGGGATGCTGTGTTTCGATTCTTCAGATGATTATACATATTT 180
                |||
Sbjct: 4382687    TTGTGCATGTCGATCTGCTGGGATGCTGTGTTTCGATTCTTCAGATGATTATACATATAT 4382746

Query: 181        CTAACAAGTTGATGATATGGTTC TTGCAGGTGATGACTGATCCTGATGCACCCAGCCCTA 240
                |||
Sbjct: 4382747    CTAACAAGTTGATGATATGGTTC TTGCAGGTGATGACTGATCCTGATGCACCCAGCCCTA 4382806

Query: 241        GTGAACCCAGAATGCGAGAGTGGGTTCATTGGTATGTACTTTTGCAGGAAAAAAAAAAGAA 300
                |||
Sbjct: 4382807    GTGAACCCAGAATGCGAGAGTGGGTTCATTGGTATGTACTTTTGCAGGAAAAAAAAAAGAA 4382866

Query: 301        GAAAAA-CACGTTGGATGATTCTTTGTGATCTAGATTTATAGTTAAGGAAAAACATAAAAC 359
                |||
Sbjct: 4382867    GAAAAAACACGTTGGATGATTCTTTGTGATCTAGATTTATAGTTAAGGAAAAACATAAAAC 4382926

Query: 360        TTAACGTTTTGCTTCTTATTGTAGGATCGTTGCGGACATTCTGGGGGCACAAACCTTAC 419
                |||
Sbjct: 4382927    TTAACGTTTTGCTTCTTATTGTAGGATCGTTGCGGACATTCTGGGGGCACAAACCTTAC 4382986

Query: 420        TCGAGGTGAAAATATATGAAACCTTACCTCTCT---CTCTCT-----GTTTTTTCGAA 469
                |||
Sbjct: 4382987    TCGAGGTGAAAAT--ATGAAACCTTACCTCTCTGATCTCTCTCTGTTTTGTTTTTTCGAA 4383044

Query: 470        AAAATCATTTGTTTCTTTACAAACTAATTTTCGACCTTCTATGATCTCTTTAATTTTCTC 529
                |||
Sbjct: 4383045    AGCATCATCTGTTTCTTTACAAACTAATTTTCGATCCTTTTATGATCTCTTTAATTTTCTC 4383104

Query: 530        TTGTTTAACTTATTCA 545
                |||
Sbjct: 4383105    TAGTTTAACTTTTTC 4383120

```

Database: Ptrichocarpa_v156_assembly_masked; 2,518 sequences; 417,137,944 total letters
scaffold 2

```

Query: 72          GTGCAAAAGATGATACCTTGAGCTTCCAAATTCATAGAGCTTCCCTCTRGGAGAGAAGAC 131
Sbjct: 11439795   GTGCAAAAGATGATACCTTGAGCTTCCAAATTCATAGAGCTTCCCTCTAGGAGAGAAGAC 11439854

Query: 132         GATAACGGCGACCTCAGCATCACAAAGAACTGATAGTTCAAAGCCTTCTTCAGKAGTCC 191
Sbjct: 11439855   GATAACAGCGACCTCAGCATCACAAAGAACTGATAGTTCAAAGCCTTCTTCAGCAGTCC 11439914

Query: 192         ATTTCTCCGCTTAGAGAAGGTGACTTGCTGCTTGAGCGTTCTCTATGCGCCTCATTTG 251
Sbjct: 11439915   ATTTCTCCGCTTAGAGAAGGTGACTTGCTGCTTGAGCGTTCTCTATGCGCCTCATTTG 11439974

Query: 252         AGTCTTTCTCTCTACCATTTCTCTTTACCTTCTCAAGCATGTGAGGACC 300
Sbjct: 11439975   AGTCTTTCTCTCTACCATTTCTCTTTACCTTCTCAAGCATGTGAAAGACC 11440023

```


8. PtSVP

Database: Ptrichocarpa_v156_assembly_masked; 2,518 sequences; 417,137,944 total letters
scaffold_2

Length = 23,562,801, Score = 649.107 bits (321), Expect = Identities = 401/434 (92%), Gaps = 7/434 (1%), Frame = +1 / +1

Query: 1 TTTTGTCTTAAATTAACAGAACCCCACTTTTGTGAGCTCCGTCTCCATGTCATTTTCATG 60|
Sbjct: 7663023 TTTTGTCTTAAATTAACAGAACCCCACTTTTCTGAGCTCCGTCTCCATGTCATTTTCATG 7663082

Query: 61 TGTCTRTTCTTTTTTGTACGGACAATGTAACGTTGTGTGTTTTTCTTTT-RAGGGAAA 119
Sbjct: 7663083 TGTCTGTTCTTTTTTGTACGGACAATGTAACGTTGTGTGTTTTTCTTTTAAAGGGAAA 7663142

Query: 120 GTATAATGGCTAGAGAGAAGATCAAGATCAAGAAGATTGACAACGTGACTGCAAGACAAG 179
Sbjct: 7663143 GTATAATGGCTAGAGAGAAGATCAAGATCAAGAAGATTGACAACGTGACTGCAAGACAAG 7663202

Query: 180 TGACCTTCTCCAAGAGGAGGCGAGGACTTTTCAAGAAAGCTGAAGAGCTTCTGTTCTTT 239
Sbjct: 7663203 TAACCTTCTCCAAGAGGAGGCGAGGCTTTTCAAGAAAGCTGAAGAGCTTCTGTTCTTT 7663262

Query: 240 GTGATGCTGAGGTTGCTGTCATCATCTTCTCTGCTACCGGCAAGCTCTTTGAGTATTCCA 299
Sbjct: 7663263 GTGATGCTGAGGTTGCTGTCATCATCTTCTCTGCTACCGGCAAGCTCTTTGAGTATTCCA 7663322

Query: 300 GCTCCAGGTACTGCWGTTTTTTTTTTACCTTTAGTGCTGARGATCTYTCTTTTTCGATTA 357
Sbjct: 7663323 GCTCCAGGTACTGCAGTTTTTTTTTTACCTTTAGTGCTGAAGATCTCTTTTATTCGATTA 7663382

Query: 358 --GTGTATGACTTCTCCTYRYGGA--CWCTATCCCWTATTTACTGTTTCTGTCTATATGC 413
Sbjct: 7663383 ATGTATATGACTTCTCCTCGCGGACTCTCTATCCCTATTTACTGTGTCTGTCTATATGC 7663442

Query: 414 TGATGYCAGTATAA 427
Sbjct: 7663443 TCATGTCACTATAA 7663456

APPENDIX 4.4 Allele frequencies

Allele frequencies of all SNPs in populations > 3 are given in this section, for each gene region. In all subsequent tables: Pop no. = population number, Al = allele, p = allele frequency of first allele (A), and q = allele frequency of second allele (a). The location of the SNPs are expressed in base pair number of region sequenced.

1.PtCDKB

Pop no.		Location of SNPs																			
		5	103	104	114	129	219	231	236	274	296	391	443	556	655	699	786	792	845		
		A/a																			
Al		T/C	T/G	A/G	G/C	T/C	G/C	G/C	C/A	C/G	G/C	T/G	T/C	T/A	A/G	C/G	T/G	C/A	G/A		
Allele frequencies																					
1	p	0.33	1	1	1	1	1	1	1	1	0.67	1	1	1	1	1	1	1	1		
	q	0.67	0	0	0	0	0	0	0	0	0.33	0	0	0	0	0	0	0	0		
5	p	0.25	1	1	1	1	1	1	1	1	0.38	1	1	1	1	1	1	1	1		
	q	0.75	0	0	0	0	0	0	0	0	0.63	0	0	0	0	0	0	0	0		
6	p	0.88	1	1	1	1	1	1	0.75	1	0.63	1	0.88	1	0.63	0.5	1	0.88	0.75		
	q	0.13	0	0	0	0	0	0	0.25	0	0.38	0	0.13	0	0.38	0.5	0	0.13	0.25		
14	p	0.25	1	1	1	1	1	1	1	1	0.25	1	1	1	1	1	1	1	1		
	q	0.75	0	0	0	0	0	0	0	0	0.75	0	0	0	0	0	0	0	0		
17	p	0.88	1	1	1	1	1	1	0.5	1	0.38	1	0.75	1	0.38	0.38	1	1	1		
	q	0.13	0	0	0	0	0	0	0.5	0	0.63	0	0.25	0	0.63	0.63	0	0	0		
18	p	0.5	1	1	1	1	1	1	1	1	0.5	1	1	1	1	0.83	1	1	1		
	q	0.5	0	0	0	0	0	0	0	0	0.5	0	0	0	0	0.17	0	0	0		
19	p	1	0.4	0	0	1	1	0	0	0.5	0	0	0	0	1	1	0	0	0		
	q	0	0.6	1	1	0	0	1	1	0.5	1	1	1	1	0	0	1	1	1		
23	p	0.7	0.6	0.6	0.6	1	0	0.6	0.6	1	0.3	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8		
	q	0.3	0.4	0.4	0.4	0	1	0.4	0.4	0	0.7	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2		
29	p	0.5	0.8	0.6	0.6	0.9	1	0.6	0.6	1	0	0.6	0.6	0.6	1	0.9	0.6	0.6	0.6		
	q	0.5	0.2	0.4	0.4	0.1	0	0.4	0.4	0	1	0.4	0.4	0.4	0	0.1	0.4	0.4	0.4		
30	p	0.33	1	1	1	1	1	1	1	1	0.17	1	1	1	1	1	1	1	1		
	q	0.67	0	0	0	0	0	0	0	0	0.83	0	0	0	0	0	0	0	0		
38	p	0.5	1	1	1	1	1	1	1	1	0.25	1	1	1	1	1	1	1	1		
	q	0.5	0	0	0	0	0	0	0	0	0.75	0	0	0	0	0	0	0	0		
41	p	0.6	1	1	1	1	1	1	1	1	0.6	1	1	1	1	1	1	1	1		
	q	0.4	0	0	0	0	0	0	0	0	0.4	0	0	0	0	0	0	0	0		

2.PtFTL1

Pop no.		Al	Location of SNPs							
			32	39	51	119	135	171	221	235
			Bases in SNP (A/a)							
		C/A	G/A	T/C	C/A	T/G	T/C	A/G	G/T	
Allele frequencies										
1	p	1	1	1	1	1	1	1	1	
	q	0	0	0	0	0	0	0	0	
5	p	0.9	1	1	0.9	1	1	0	1	
	q	0.1	0	0	0.1	0	0	1	0	
6	p	0.9	1	1	0.9	1	1	0.20	1.00	
	q	0.1	0	0	0.1	0	0	0.80	0.00	
11	p	0.9	1	1	1	1	1	0.90	1.00	
	q	0.1	0	0	0	0	0	0.10	0.00	
14	p	0.8	1	1	0.9	1	1	1	1	
	q	0.2	0	0	0.1	0	0	0	0	
17	p	1	1	1	1	1	1	1.00	1.00	
	q	0	0	0	0	0	0	0.00	0.00	
18	p	1	1	1	1	1	1	1.00	1.00	
	q	0	0	0	0	0	0	0.00	0.00	
19	p	0.9	1	1	0.9	0.5	0.5	1.00	1.00	
	q	0.1	0	0	0.1	0.5	0.5	0.00	0.00	
20	p	1	0.7	0.6	0.9	1	1	1.00	1.00	
	q	0	0.3	0.4	0.1	0	0	0.00	0.00	
23	p	1	1	1	1	1	1	1.00	1.00	
	q	0	0	0	0	0	0	0.00	0.00	
29	p	0.9	1	1	1	1	1	0.80	0.60	
	q	0.1	0	0	0	0	0	0.20	0.40	
30	p	0.8	1	1	0.8	1	1	0.60	1.00	
	q	0.2	0	0	0.2	0	0	0.40	0.00	
38	p	1	1	1	1	1	1	1	1	
	q	0	0	0	0	0	0	0	0	
41	p	0.5	1	1	0.83	1	1	1	1	
	q	0.5	0	0	0.17	0	0	0	0	

3.PTLF

Pop no.		Location of SNPs																		
		1	11	31	58	82	106	116	122	123	117	150	158	164	193	258	281	310	314	318
		Bases in SNP (A/a)																		
Al		A/T	C/G	T/A	G/T	C/A	T/C	T/C	A/T	C/G	C/T	A/G	C/T	G/C	A/T	A/G	G/A	T/A	T/C	
Allele frequencies																				
1	p	0.83	1	1	0.83	1	1	0.67	1	1	1	1	1	0.83	0.67	1	1	1	1	
	q	0.17	0	0	0.17	0	0	0.33	0	0	0	0	0	0.17	0.33	0	0	0	0	
5	p	0.63	1	1	0.5	1	1	0.5	1	1	1	1	1	0.5	0.5	1	1	1	1	
	q	0.38	0	0	0.5	0	0	0.5	0	0	0	0	0	0.5	0.5	0	0	0	0	
6	p	1	1	0.5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	q	0	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
11	p	0.5	1	0.7	0.7	0.9	1	0.5	1	0.9	0.9	1	0.9	1	0.6	0.7	1	1	1	0.5
	q	0.5	0	0.3	0.3	0.1	0	0.5	0	0.1	0.1	0	0.1	0	0.4	0.3	0	0	0	0.5
14	p	0.6	1	0.7	1	0.8	1	0.6	1	0.7	0.8	1	0.7	1	1	1	0	1	1	0.2
	q	0.4	0	0.3	0	0.2	0	0.4	0	0.3	0.2	0	0.3	0	0	0	1	0	0	0.8
17	p	0.7	1	0.6	1	1	1	0.7	1	1	1	1	0.7	0.7	1	1	1	1	1	0.9
	q	0.3	0	0.4	0	0	0	0.3	0	0	0	0	0.3	0.3	0	0	0	0	0	0.1
18	p	0.5	1	1	1	0.6	1	0.4	1	0.6	0.6	1	0.6	1	0.9	0.8	0	1	1	0.4
	q	0.5	0	0	0	0.4	0	0.6	0	0.4	0.4	0	0.4	0	0.1	0.2	1	0	0	0.6
19	p	0.1	0.6	1	1	1	0.3	0.1	0.5	1	1	0.5	1	1	0.6	0.9	0.2	0.2	0.2	0.9
	q	0.9	0.4	0	0	0	0.7	0.9	0.5	0	0	0.5	0	0	0.4	0.1	0.8	0.8	0.8	0.1
23	p	0.5	1	1	0.7	0.8	1	0.5	1	0.8	1	1	0.8	1	0.7	0.7	0.6	1	1	0.6
	q	0.5	0	0	0.3	0.2	0	0.5	0	0.2	0	0	0.2	0	0.3	0.3	0.4	0	0	0.4
29	p	0.13	0.88	0.88	0.88	0.5	0.88	0.88	0.88	0.75	1	0.88	0.63	0.88	0.75	0.88	1	0.75	0.75	0.75
	q	0.88	0.13	0.13	0.13	0.25	0.13	0.13	0.13	0.25	0	0.13	0.38	0.13	0.25	0.13	0	0.25	0.25	0.25
30	p	0.83	1	0.83	1	0.83	1	0.83	1	0.83	0.83	1	0.83	1	1	1	1	0.33	1	0.67
	q	0.17	0	0.17	0	0.17	0	0.17	0	0.17	0.17	0	0.17	0	0	0	0	0.67	0	0.33
38	p	0.5	1	0.8	0.5	1	1	0.5	1	1	1	1	1	1	0.5	0.5	0.6	1	1	0.8
	q	0.5	0	0.2	0.5	0	0	0.5	0	0	0	0	0	0	0.5	0.5	0.4	0	0	0.2
41	p	0.5	1	0.6	1	0.4	1	0.5	1	0.5	0.5	1	0.5	1	1	1	0	1	1	0
	q	0.5	0	0.4	0	0.6	0	0.5	0	0.5	0.5	0	0.5	0	0	0	1	0	0	1

4.PtMFT

Pop no.		Location of SNPs															
		9	38	57	77	85	107	193	356	391	408	588	629	650	689	746	778
		Bases in SNP (A/a)															
		T/G	A/G	A/G	C/A	A/C	G/A	T/C	A/G	G/C	C/A	A/G	A/T	A/C	G/T	G/A	A/G
		Allele frequencies															
5	p	1	0.50	1.00	0.50	0.00	0.50	1.00	1	1	0.25	1	1	1	1	1	1
	q	0	0.50	0.00	0.50	1.00	0.50	0.00	0	0	0.75	0	0	0	0	0	0
6	p	0	1	0	1	1	1	1	1	0.4	1	0.3	0	0.3	1	0	0
	q	1	0	1	0	0	0	0	0	0.6	0	0.7	1	0.7	0	1	1
11	p	0.9	0.6	0.9	0.6	0.3	0.5	0.7	0.5	1	1	1	0.6	1	0.7	0.5	1
	q	0.1	0.4	0.1	0.4	0.7	0.5	0.3	0.5	0	0	0	0.4	0	0.3	0.5	0
14	p	0.75	0.75	0.75	0.38	0.38	0.75	1	1	0.88	1	0.5	0.38	1	1	0.5	0.5
	q	0.25	0.25	0.25	0.63	0.63	0.25	0	0	0.13	0	0.5	0.63	0	0	0.5	0.5
18	p	0.67	0.83	0.67	0.33	0.33	1	1	1	1	1	0.67	0.33	0.67	1	0.67	0.33
	q	0.33	0.17	0.33	0.67	0.67	0	0	0	0	0	0.33	0.67	0.33	0	0.33	0.67
23	p	0.67	1	0.5	1	1	1	1	1	1	1	1	1	1	1	1	1
	q	0.33	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0
29	p	0.63	0.38	0.88	0.5	0.13	0.63	1	1	1	1	0.75	0.25	1	1	0.25	0.75
	q	0.38	0.63	0.13	0.5	0.88	0.38	0	0	0	0	0.25	0.75	0	0	0.75	0.25
30	p	1	1	1	0.33	0	0.67	1	1	1	1	1	1	0.67	1	0	0.67
	q	0	0	0	0.67	1	0.33	0	0	0	0	0	0	0.33	0	1	0.33
38	p	0.83	1	0.83	0.5	0.67	0.5	0.5	1	1	1	1	1	1	1	1	1
	q	0.17	0	0.17	0.5	0.33	0.5	0.5	0	0	0	0	0	0	0	0	0

5.PtSOC1

Pop no.		Location of SNPs						
		9	53	60	72	120	186	301
		Bases in SNP (A/a)						
		T/C	G/A	A/C	G/T	A/G	T/G	A/G
		Allele frequencies						
1	p	0.5	1	1	1	1	1	1
	q	0.5	0	0	0	0	0	0
5	p	1	1	1	1	1	1	1
	q	0	0	0	0	0	0	0
6	p	1	1	1	1	1	0.5	1
	q	0	0	0	0	0	0.5	0
11	p	0.9	0.9	1	0.9	1	1	1
	q	0.1	0.1	0	0.1	0	0	0
14	p	0.5	1	1	1	0.9	0.9	0.9
	q	0.5	0	0	0	0.1	0.1	0.1
17	p	1	1	0.9	0.9	0.9	0.6	0.8
	q	0	0	0.1	0.1	0.1	0.4	0.2
18	p	1	1	1	1	0.88	0.88	0.88
	q	0	0	0	0	0.13	0.13	0.13
19	p	1	0.5	0.5	1	1	1	0.5
	q	0	0.5	0.5	0	0	0	0.5
20	p	1	0.3	0.9	0.9	1	0.9	0.5
	q	0	0.7	0.1	0.1	0	0.1	0.5
23	p	1	0.88	1	1	1	1	0.88
	q	0	0.13	0	0	0	0	0.13
29	p	1	0.5	1	1	1	1	1
	q	0	0.5	0	0	0	0	0
30	p	1	1	0.9	0.9	1	0.9	0.8
	q	0	0	0.1	0.1	0	0.1	0.2
38	p	0.5	1	1	0.88	0.5	1	1
	q	0.5	0	0	0.13	0.5	0	0
41	p	1	1	1	1	1	0	1
	q	0	1	2	3	4	5	0

6.PtSVP

Pop no.		Al	Location of SNPs											
			33	66	112	314	338	344	374	375	376	381	389	419
			Bases in SNP (A/a)											
			C/T	A/G	G/A	A/T	A/G	C/T	C/T	G/A	C/T	T/A	A/T	T/C
Allele frequencies														
1	p	1	1	1	1	1	1	1	1	0	1	1	1	
	q	0	0	0	0	0	0	0	0	1	0	0	0	
5	p	1	1	1	1	1	1	1	1	1	1	1	0.5	
	q	0	0	0	0	0	0	0	0	0	0	0	0.5	
6	p	1	1	1	1	1	1	1	1	0.6	1	1	0.4	
	q	0	0	0	0	0	0	0	0	0.4	0	0	0.6	
11	p	1	0.9	1	1	1	1	1	1	0.5	1	1	1	
	q	0	0.1	0	0	0	0	0	0	0.5	0	0	0	
14	p	1	1	1	1	1	1	1	1	0.7	1	1	0.7	
	q	0	0	0	0	0	0	0	0	0.3	0	0	0.3	
17	p	1	1	1	1	1	1	1	1	0.5	1	1	1	
	q	0	0	0	0	0	0	0	0	0.5	0	0	0	
18	p	1	1	1	1	1	1	1	1	0.63	0.88	1	0.88	
	q	0	0	0	0	0	0	0	0	0.38	0.13	0	0.13	
19	p	1	0	0	0	0.5	0	0.5	1	1	1	0	1	
	q	1	1	1	1	0.5	1	0.5	0	0	0	1	0	
30	p	1	1	1	1	1	1	1	1	1	1	1	1	
	q	0	0	0	0	0	0	0	0	0	0	0	0	
38	p	1	1	1	1	1	1	1	1	0.67	0.67	1	0.67	
	q	0	0	0	0	0	0	0	0	0.33	0.33	0	0.33	