Adaptive genetic variation in Scots pine (*Pinus sylvestris* L.) in Scotland

Matti J. Salmela

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Declaration

I hereby declare that this thesis is composed of work carried out by myself unless otherwise acknowledged and that it has not in whole or in part been previously presented for any other degree or professional qualification.

Matti Salmela

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it, you probably never did). I'm missing our regular and therapeutic pub nights already, partly because I have to pay a lot more for my pints here in Finland. Emily Barlow, James Ryder, Sanna Kivimäki, and Lorna Wilkie must be specifically mentioned since they've had to put up with me most. I enjoyed being in the 'Room of Doom' with you guys!

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Toivottavasti ei aivan päin mäntyä mennyt tämäkään urakka.

"Pitkäaikainen käyttö ja yliannokset voivat aiheuttaa painostavan olon ja verenpaineen laskua."

- mäntyperäisten luontaisrohtojen käyttöön liittyviä riskejä

Abstract

Genetic differentiation in phenotypic traits among populations from heterogeneous environments is often observed in common-garden studies on forest trees, but data on adaptive variation in Scots pine (*Pinus sylvestris* L.) in Scotland are limited. As a result, current seed transfer guidelines are based on earlier molecular marker studies and do not take into account environmental or adaptive genetic variation. An analysis of spatial variation in climate showed substantial differences in temperature and precipitation among the native Scots pine sites in Scotland. To investigate whether differentiation in response to environmental variation has occurred in Scotland, a glasshouse-based common-garden trial of ~3,360 seedlings from 21 populations and 84 open-pollinated families was established in 2007. At the beginning of the 2nd growing season, timing of bud flush showed evidence of genetic differentiation among populations, with those from cooler origins generally flushing earlier. Variation was also found among families within populations, suggesting that the trait is genetically controlled. Populations and families showed different levels of variability in this trait which could be partly due to variable levels of temporal climate fluctuation in different parts of Scotland. Chlorophyll fluorescence was used to examine drought response in three-year old seedlings from five populations on sites that experience contrasting levels of annual rainfall. It was found that the response was not related to rainfall, but possibly to more complex moisture variables that also take into account additional factors such as evaporation. Also, photosynthetic capacity in response to cold winter temperatures varied significantly among eight populations that were kept outdoors, and the largest reduction was seen in seedlings from the mildest, most maritime coastal site. The following spring, height growth and needle flush started earlier in seedlings from cooler locations. Earlier studies on genetic diversity of native pinewoods have shown high levels of selectively neutral variation in this predominantly outcrossing conifer, and a mating system analysis with a limited number of microsatellite markers supported this pattern. Together, these data suggest that despite significant historic population size decrease, environmental gradients have resulted in genetic differentiation among native pinewoods. In order to minimise the risk of planting poorly-adapted stock and

to maximise the success of replanting programmes, it is important that the origins of planting stock are carefully considered in management guidelines for the species.

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1.1.1 What is local adaptation?

Many organisms occur in environments that vary in space, causing spatially varying selection pressures (Kawecki and Ebert 2004). In response to such variation, populations have two options for survival: 1) phenotypic plasticity, which allows single genotypes to produce optimal phenotypes in different environments, or 2) adaptive genetic differentiation due to different alleles (or allele frequencies/combinations) being favoured in different environments. Distinguishing between these two factors is possible via an examination of local adaptation.

Local adaptation is defined as a phenomenon where natural selection has caused genetic differentiation among populations and where a population has higher relative fitness at its home site compared to transplanted populations, i.e., there is a genotype × environment interaction (Kawecki and Ebert 2004). If locally adapted, a population's fitness at other, environmentally different sites will be lower than that of local populations, and the stronger the divergent selection, the more likely local adaptation is thought to be. However, gene flow can counteract genetic differentiation and adaptation, allowing an influx of genetic variation from sites where different allelic make-ups might have been favoured by natural selection. This has been suggested as one factor that might cause range limits in different species: if peripheral populations receive high levels of gene flow from other parts of the distribution, they might not be able to reach their optimum level of adaptation (García-Ramos and Kirkpatrick 1997). In a model by Kirkpatrick and Barton (1997), populations can reach their trait optimum despite gene flow if population size stays the same across an environmental gradient, but if population size decreases towards

range peripheries and if gene flow occurs between populations, marginal populations are expected to deviate from their optimum phenotype. However, the model assumes constant genetic variance across the gradient: an assumption that could be violated in natural populations (Bridle and Vines 2007). If no selection is operating, gene flow is expected to homogenise adaptive variation among populations (Kawecki and Ebert 2004), but on the other hand, if progeny from matings between local and foreign parents have lower fitness than those with two local parents, they will have a poorer chance of survival and might not make it to adulthood, thus maintaining the local adaptive genetic architecture (e.g. Burczyk, DiFazio et al. 2004; Kawecki and Ebert 2004). Other factors also can influence adaptation processes: random genetic drift can erase much of the adaptive variation in small populations with low effective population size, while lack of genetic variation in critical traits can prevent adaptation even in the presence of selection pressure (Willi, Van Buskirk et al. 2006). Spatial heterogeneity, genetic differentiation, and gene flow among populations can promote the maintenance of genetic variation across populations, both at a molecular (Hedrick 1986) and quantitative trait level (Slatkin 1978; Barton 1999), while temporal variation is often thought result in generalist phenotypes (e.g. Kawecki and Ebert 2004).

1.1.2 Local adaptation in plants

In plants, studies of patterns of local adaptation have a long history due to the importance of many species in agriculture or forestry (Linhart and Grant 1996). Plants are sessile and as the distributions of many species cover heterogeneous environments, they cannot escape selection pressure and must therefore adapt to their home environments and/or maintain phenotypic plasticity. In order to explore the causes of genetic differentiation among populations and to distinguish between drift and adaptation, it is useful to understand how environmental factors vary spatially among sampled sites and whether their variation is associated with phenotypic differences (Kawecki and Ebert 2004). Adaptation in plants can occur in response to abiotic factors such as soil, moisture, and temperature conditions and biotic factors

such as pests or herbivores. The scale of differentiation is influenced by selection intensity, the level of gene flow, and a species' life history characteristics (Linhart and Grant 1996). As the patterns of co-variation and the spatial scale of variation among different biotic and abiotic factors might be different, the scale of genetic differentiation between different adaptive traits can vary, too.

Adaptive differentiation is reflected in associations between environmental factors and patterns of quantitative trait variation observed when different populations are grown under homogeneous common-garden conditions. In such a design, variation in phenotype (P) is usually assumed to be due to genotypic variation (G) at genes controlling the trait, while environmental contribution (E) is thought to be minor (e.g. White, Adams et al. 2007). However, differences observed in quantitative traits under common-garden conditions do not confirm local adaptation because the fitness advantage of native populations cannot be assessed (Kawecki and Ebert 2004). A recent meta-analysis of reciprocal transplant experiments in 32 species suggested that local adaptation in herbaceous plants is less common than thought, as in only 45.3% of the cases the local population outperformed the foreign one in both compared environments and in 51.4% of the cases one population performed best at both sites (Leimu and Fischer 2008). In this dataset, local adaptation was more common in large populations, suggesting that the amount of adaptive genetic variation might be low in small populations. However, these observations are specific to the studies included in the meta-analysis and to the populations and sites covered.

An often used genetic approach to separate the effects of selection and drift is to compare differentiation in quantitative traits (Q_{ST}), which can be influenced by both demography and selection, to that of selectively neutral molecular markers (F_{ST}) whose variation is thought to reflect demographic processes (Spitze 1993). Q_{ST} is estimated as $V_P/(V_P+2V_A)$, where V_P is the among-population variance component and V_A is the additive genetic variance (within-population component that can be estimated from the variance due to families within populations). A family-structured common-garden trial is required for estimating these components (McKay and Latta 2002). Genetic variation in neutral markers can be estimated by assessing variation in

molecular markers such as allozymes or microsatellites, and F_{ST} can then be expressed as $1-(H_S/H_T)$, where H_S is the within-population diversity and H_T is the total diversity (Frankham, Ballou et al. 2002). If Q_{ST} and F_{ST} indices are similar, differentiation in a quantitative trait is thought to be due to drift, but if Q_{ST} is larger than F_{ST}, at least some of this difference is considered to be due to divergent selection (Merilä and Crnokrak 2001). For example, Willi, Van Buskirk et al. (2007) found that in *Ranunculus reptans* L. growing in populations of different sizes around a lake in Central Europe, Q_{ST} and F_{ST} were higher among small populations which suggests that while drift might have had a bigger role in shaping variation in small populations, it has not fully overcome the effects of divergent selection. However, estimating both Q_{ST} and F_{ST} is based on many assumptions and can be problematic. Estimates of V_A are needed for calculating Q_{ST}, and these can be obtained by using for instance parent-offspring regressions or half-sib progeny designs (Falconer and Mackay 1996). However, some of the variation can also be due to additional factors such as dominance or maternal effects (Leinonen, O'Hara et al. 2008; Whitlock 2008), and different V_A estimates might be obtained in contrasting environments (Hoffmann and Merilä 1999). Furthermore, the statistical properties of Q_{ST} are poor, especially in studies with only a small number of populations (O'Hara and Merilä 2005). Traditional ways to estimate F_{ST} can be problematic when using highly variable genetic markers which increase the within-population diversity and can thus result in a very low F_{ST} even if populations are fully differentiated (Hedrick 1999). It is also misleading to use only mean F_{ST} because the distributions of locus-specific estimates can be large (Whitlock 2008). If environmental data for the sampled populations is available, complex Q_{ST}-F_{ST} comparisons can be avoided simply by comparing mean phenotypes to environmental variables such as mean temperature or moisture. Significant associations are good evidence for adaptive differentiation even if F_{ST} is not estimated.

1.1.3 Local adaptation in trees

Forest trees are among the most intensively studied plant species, and population (provenance) transfers have been carried out by foresters in many species for over 200 years (Mátyás 1996; Savolainen, Pyhäjärvi et al. 2007). By the 18th century, small-scale Scots pine (*Pinus sylvestris* L.) experiments had been carried out in Finland, and sourcing of quality oak and pine seed stock was becoming an important issue to European shipyards (Wright 1976; Mátyás 1996). The oldest Scots pine (*Pinus sylvestris* L.) experiment with good documentation was done by de Vilmorin near Paris starting in 1821 where he grew seedlings originating from the northern part of the continent (Wright 1976). Before his work, differences in provenance performance were thought to be due to variation in growing conditions, but publication of his work contributed to the development of understanding of the genetic basis of inheritance. After de Vilmorin's work, similar experiments were started in other parts of Europe and the first replicated pine tests were started by the International Union of Forest Research Organizations (IUFRO) in 1908. The NC-99 trial of Scots pine, for example, included 170 populations from Europe and Asia grown at a number of locations in United States (Wright, Pauley et al. 1966). The main motivation for these experiments was to find the best-growing seed sources for different sites (Mátyás 1996; Savolainen, Pyhäjärvi et al. 2007; Aitken, Yeaman et al. 2008), but they have also been of interest to evolutionary biology studies when well-documented and replicated because of their extensive sampling across large geographic areas. In many of these studies adaptive genetic variation has been found to be clinal rather than manifested as well-defined ecotypes (Langlet 1959). More recently, the importance of understanding patterns of adaptive variation in species of primarily ecological value has been recognized (e.g. Bower and Aitken 2008).

Patterns of quantitative trait variation in trees

Many forest tree species have ranges covering wide geographic and environmentally diverse areas. For example, the range of Douglas-fir (*Pseudotsuga menziesii* (Mirb.)

Franco) covers a vast part of western North America, both coastal and inland areas to altitudes of 3,500 m (e.g. St. Clair, Mandel et al. 2005). Common-garden studies on trees from the northern hemisphere, where environmental conditions for growth are ideal only for a limited amount of time each year, have frequently reported population differences in traits related to phenology, growth, and stress tolerance, and commonly shorter periods of active growth and earlier development of cold hardiness in seedlings are associated with shorter growing seasons and earlier onset of cold temperatures at the populations' origin (reviewed in Howe, Aitken et al. 2003; Savolainen, Pyhäjärvi et al. 2007). In environments where spatial heterogeneity is very high, genetic differentiation can occur at distances as short as 100-200 m (Campbell 1979). Less is known for instance about below-ground adaptations to soil, although in Scots pine, root length was found to be associated with average annual temperature at the populations' home sites among 45 Eurasian populations (Brown 1969). However, not all phenotypic differences under common-garden conditions are necessarily due to a simple Mendelian inheritance of additive parental alleles: in Norway spruce (*Picea abies* (L.) Karst.), phenotypic variation in adaptive traits has been shown to be influenced by differences in temperature conditions experienced by seed while maturing in their native environments (Kvaalen and Johnsen 2008). For instance, when trees from northern Norway were moved to a southern seed orchard, their progeny was phenotypically similar to those of local parents (Skrøppa, Kohmann et al. 2007). It is not yet well-established whether such maternal effects are a common feature among other forest trees (Rohde and Junttila 2008).

Studying adaptive differentiation and local adaptation in trees

Common-garden experiments

The benefit of common-garden studies on young seedlings is that large sample sizes can be maintained in a small space, environmental conditions can be easily controlled and monitored, and many quantitative traits can be quickly scored (Johnson, Sorensen et al. 2004). Despite the longevity of trees, limiting studies to seedlings can be justified as selection in trees is thought to operate most efficiently at very early developmental stages (Persson and Ståhl 1990; Petit and Hampe 2006) and provided that variation is due to a Mendelian inheritance of genetic variation and not, for instance, to maternal effects. Thus, common-garden experiments can be used to study how adaptive traits of different populations vary under experimental conditions, but they cannot be used to assess effects of population transfers along environmental gradients or how trait variation is influenced by different growing conditions (unless the experiment is replicated in multiple environments).

Complex trait variation results from both genetic and environmental factors (Falconer and Mackay 1996), and therefore, a trait's expression can be different across contrasting environments (e.g. Hoffmann and Merilä 1999; Conner, Franks et al. 2003). Similar observations have also been documented in trees: in Douglas-fir, quantitative trait variation can be associated with different environmental factors in different soil and air temperature treatments (Campbell and Sorensen 1978). In nature, individuals in a population are not exposed to exactly the same environment every year and for example, boreal forests are characterised by extensive inter- and intra-annual temperature variation (Bonan and Shugart 1989), meaning that the patterns of complex trait variation can also vary temporally. This factor has not been extensively studied in forest trees, and often data is collected over one year only. In provenance trials, cumulative effects over many years are usually assessed, rather than those specific to a particular year. However, temporal variation has been demonstrated in some traits. For instance at two sites in southern and south-eastern

Finland, heat sum requirement for bud flush among Scots pine populations from different latitudes in Finland and Russia varied over three years, although the ranking of populations remained similar each year (Beuker 1994). This variation could be due to timing of bud flush being determined by chilling and heat sum requirements (Aitken and Hannerz 2001), and it has been shown in Scots pine that differences in heat sum requirement among populations can be decreased by extended chilling (Leinonen 1996). This suggests that timing of bud flush could vary between years as a result of interaction between these two temperature factors. First-year growth cessation has also been shown to be affected by both photoperiod and heat sum (Koski and Sievänen 1985), and temporal climate fluctuations are reflected in tree ring variation (Hughes, Schweingruber et al. 1984).

Reciprocal transfer trials

Whilst testing for local adaptation requires an experimental design in which a local population's performance can be compared to that of those from other locations (Kawecki and Ebert 2004), such experiments in long-lived trees are laborious, time-consuming, expensive and thus, normally established only for species of commercial importance (Mátyás 1996; González-Martínez, Krutovsky et al. 2006). Moreover, because seedlings used in these experiments are usually grown under nursery conditions during their first few years and then transferred to tended field sites, patterns of variation observed at trial sites might not reflect the outcome of various processes (e.g. competition and early juvenile selection) occurring in natural conditions (Aitken, Yeaman et al. 2008). Transfer trials established for commercially important tree species such as Scots pine (Eiche 1966; Shutyaev and Giertych 1998) and lodgepole pine (*Pinus contorta* Douglas; Rehfeldt, Ying et al. 1999) have indicated that populations often grow best in their home environments and that transfers along environmental gradients influence survival and growth (Eriksson, Andersson et al. 1980; Persson and Ståhl 1990). Although these patterns are generally compatible with local adaptation, phenotypic plasticity, where trees survive outside their native environment despite worse performance than local seedlings, is

also a common feature of trees (Mátyás 1996; Aitken, Yeaman et al. 2008). In some studies on North American and Eurasian pines, it has been reported that peripheral populations occupy non-optimal climates and that their performance could be improved by transferring them to milder environments (Rehfeldt, Ying et al. 1999; Rehfeldt, Tchebakova et al. 2002; Savolainen, Pyhäjärvi et al. 2007). This is often assumed to be due to strong gene flow from range centres, but this interpretation is also based on an assumption that the environments are temporally stable across the ranges. If temporal variability increases towards range peripheries, the observed patterns could also be due to adaptation to fluctuating environmental factors. However, no temporal climate variation analyses have been carried out to explore this hypothesis.

Management of adaptive genetic resources in forest trees

An understanding of how different populations have adapted to their native environments is essential for the development of seed transfer guidelines that define areas within which seed stock can be moved with a relatively small risk of maladaptation (Ying and Yanchuk 2006). Such guidelines can aim at either maximising growth potential at each site (forestry species), or at maintaining natural patterns of variation when, for instance, expanding or restoring existing woodlands (McKay, Christian et al. 2005). For example in British Columbia, original seed zones for Douglas-fir were based on general climate features and ecological classification and divided the area into over 60 zones (Ying and Yanchuk 2006). Studies on the patterns of adaptive variation in the species showed that populations were differentiated in many quantitative traits and that these patterns were related to longitudinal, latitudinal, and altitudinal gradients. Such observations were then used to estimate the impacts of population transfers along these gradients and to scale the size of seed zones (e.g. Campbell 1986). The concept of floating seed zones was introduced following studies showing that analogous adaptations can arise in environments that are similar but not necessarily close geographically (e.g. Rehfeldt 1991). Therefore, this approach is based on matching planting site and seed source

environments and has become the main approach used for instance in British Columbia (Ying and Yanchuk 2006). Availability of climate data has also enabled relating the patterns of adaptive variation to specific temperature or precipitation variables rather than just their geographical surrogates (Rehfeldt, Ying et al. 1999). However, adaptations occur at different scales in different species and as a result seed zones for instance in the Pacific Northwest are species-specific and thus variable in size (Johnson, Sorensen et al. 2004). In Britain, four provenance regions and 24 seed zones are currently recognized for all native species with the exception of Scots pine, but the zones are not based on patterns of adaptive variation in these tree species (Hubert and Cottrell 2007).

Molecular marker diversity in trees

A common observation in a wide range of species is that the distribution of neutral molecular marker and quantitative trait variation among and within populations varies and that molecular markers do not accurately predict quantitative trait differentiation (McKay and Latta 2002). Also in trees, among-population differentiation at neutral nuclear markers is often noticeably less than in quantitative traits (e.g. Yang, Yeh et al. 1996). These patterns in marker diversity are usually attributed to the efficient wind-mediated mixing of pollen pools even among distant populations and high outcrossing rates (Hamrick, Godt et al. 1992; Hamrick 2004; White, Adams et al. 2007; Williams 2010), while divergent selection maintains differentiation in adaptive traits (Howe, Aitken et al. 2003; Savolainen, Pyhäjärvi et al. 2007). Conifers generally have a mixed mating system, but many species are characterised by outcrossing rates above 0.9 (Mitton 1992, Williams 2009). However, small differences at nuclear molecular markers among populations of adult trees do not necessarily mean strong current levels of gene flow (Sork, Nason et al. 1999), and to assess landscape-level processes, alternative approaches are needed. For instance, progeny arrays in multiple parents resulting from pollination events in the same year can be genotyped to explore the genetic structure of pollen pools sampled by different mothers (Smouse, Dyer et al. 2001). Such studies on real-time

gene flow in tree populations have shown that generally the majority of fertilizing pollen is of local origin, but also that long-distance gene flow from other sites can account for a significant proportion of the matings (Smouse and Sork 2004).

In addition to nuclear genomes, plants also have haploid mitochondrial and chloroplast genomes which are mainly maternally inherited (i.e., transmitted via seed) and the markers of which often show higher levels of among-population differentiation due to the smaller effective population size of these genomes and more restricted seed flow (Petit, Kremer et al. 1993; Ennos 1994). However, in gymnosperms, paternal (pollen-transmitted) inheritance of chloroplasts has been reported (Neale and Sederoff 1989). Due to the more restricted spatial distribution of uniparentally-inherited polymorphisms, such markers have become frequently-used tools for examining for instance the postglacial colonization histories of European plant species (e.g. Taberlet, Fumagalli et al. 1998). In many European tree species, the highest levels of marker diversity have been found in Central Europe which could be due to ancient refugia or an admixture of genetically-differentiated lineages (Petit, Aguinagalde et al. 2003).

Genetic basis of quantitative trait variation

Although phenotypic assessments can be used to show genetic differentiation in quantitative traits, they do not provide any information on the genes causing phenotypic variation (González-Martínez, Krutovsky et al. 2006). Understanding the molecular background of complex trait variation has become an active research area in a variety of plants (Siol, Wright et al. 2010) and also in trees (Neale and Ingvarsson 2008). Long breeding cycles in trees could be shortened if the loci contributing to traits such as cold adaptation were known and if their variation explained a sufficient proportion of variation in phenotype, enabling phenotypic prediction based on genotype (Howe, Aitken et al. 2003). Quantitative trait loci (QTL) mapping experiments using known pedigrees have discovered genomic regions associated with adaptive traits in a number of species, but the resolution of

the studies has not allowed specific loci to be distinguished (Howe, Aitken et al. 2003; Savolainen, Pyhäjärvi et al. 2007) and such studies are also known to underestimate the number of QTL and overestimate their effects (Barton and Keightley 2002). Nowadays, mapping studies are more often based on nucleotide variation in known candidate genes (Wright and Gaut 2005). Association mapping, which aims to find relationships between polymorphisms and quantitative variation in natural populations, has been suggested as a powerful tool for trees due to their undomesticated populations and quick decay of linkage disequilibrium (Neale and Savolainen 2004). This means that in theory, causative polymorphisms could be mapped at a very fine resolution. However, although modern genomic approaches enable assessment of genetic differentiation simultaneously in a large number of loci (González-Martínez, Krutovsky et al. 2006), the influence of polymorphisms on adaptive variation cannot be evaluated without phenotypic data. Furthermore, in addition to selection, demographic events such as bottlenecks and range expansions can influence the patterns of genomic variation and mimic the effects of selection (Wright and Gaut 2005). Therefore, understanding genome-wide patterns of variation is required to allow differentiation of selection and demography (Savolainen and Pyhäjärvi 2007). Interestingly, while the generation of genomic data has accelerated rapidly in evolutionary biology, human disease mapping, and animal and plant breeding, obtaining phenotypic data at a similar rate has become an obstacle (Houle 2010).

Candidate gene studies have already been carried out in species such as Douglas-fir (Eckert, Bower et al. 2009), Sitka spruce (*Picea sitchensis* (Bong.) Carr.; Holliday, Ritland et al. 2010), and aspen (*Populus tremula* L.; Ma, Hall et al. 2010), and the current evidence from both QTL experiments and genomic studies points to a complex polygenic inheritance of adaptive traits. Indeed, theoretical studies have shown that significant phenotypic differentiation can be achieved with minor changes at underlying loci (Latta 1998; Le Corre and Kremer 2003), which can complicate their discovery in mapping studies if sample sizes are not large enough. So far, causative polymorphism have mainly been searched for within coding regions of genes, and it remains to be seen what proportion of phenotypic variation is due to

gene expression (e.g. polymorphisms in *cis-* or *trans-*acting control regions). Patterns of genomic diversity have generally been interpreted in the context of demographic factors such as historic bottlenecks and range expansions (Lascoux, Pyhäjärvi et al. 2008), but so far, the possibility of genetic structures within populations and their influence on genomic variation has not been considered in detail. Recently, the importance of such considerations has been recognized (Jansson and Ingvarsson 2010), and in Sitka spruce for instance, peripheral populations have been shown to be more structured than those from more central parts of the range (Gapare and Aitken 2005). Inferring demographic histories of populations from genomic data could lead to false interpretations if populations vary in the level of substructuring and if this is not taken into account in the analyses.

1.2 Case study: adaptation in Scots pine in Scotland

Scots pine (*Pinus sylvestris* L., family Pinaceae) is a long-lived conifer and the only pine species native to northern Europe. It has one of the widest distributions of all conifers, extending from northern Finland to Turkey and from western Spain to eastern Siberia (figure 1.1, Critchfield and Little 1966), covering a huge range of environments and altitudes from sea level to over 2,000 metres. In many countries Scots pine is also a commercially important timber species, and its wood is being used for construction, furniture, and other products. In North America, it has been extensively planted due to its popularity as a Christmas tree. In Scotland, the species is considered a national icon and is a foundation species in the Caledonian forest. Today, native pinewoods recognized by the Forestry Commission constitute only less than 1% of the species' maximum postglacial range (Mason, Hampson et al. 2004) and represent the only recognised UK resource for this habitat, Caledonian pinewood, which receives protection under the European Commission Habitats directive.
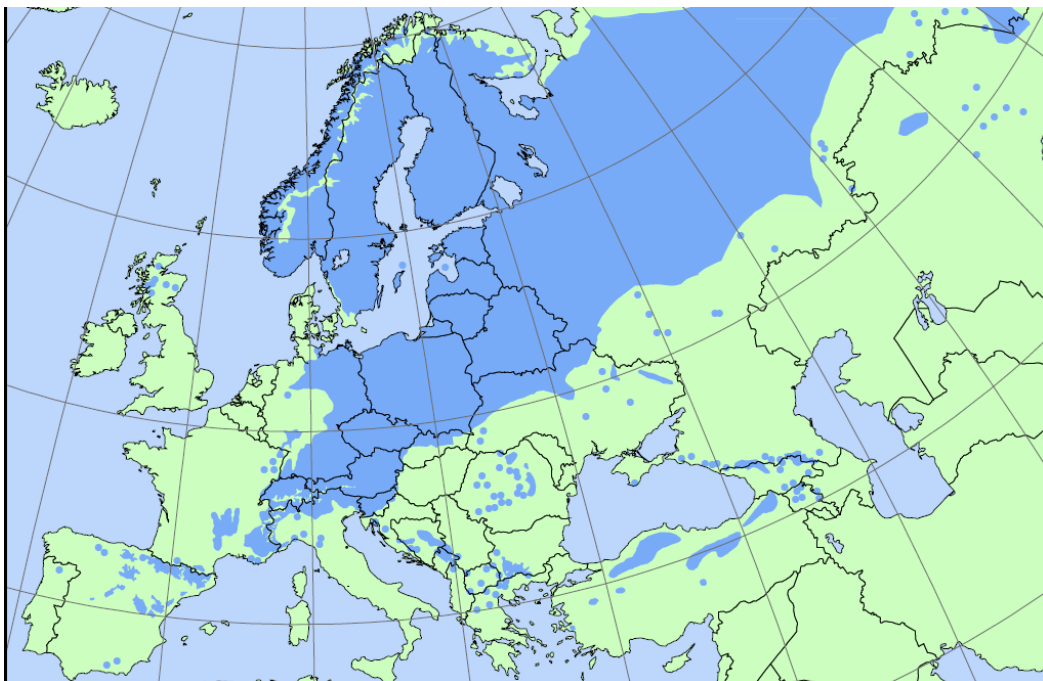
Figure 1.1 Distribution of Scots pine in Europe (source: **http://www.euforgen.org**).

1.2.1 Evolutionary history of Scots pine in Scotland

The last glaciation has strongly influenced the distributions of numerous species in Europe as, during the last glacial maximum 23 000 – 18 000 years ago, ice covered the majority of northern Europe (Svendsen, Astakhov et al. 1999). Pine survived through the ice age in the Iberian, Italian, and Balkan peninsulas (Bennett, Tzedakis et al. 1991), but macrofossil evidence for refugia has also been found in central parts of Europe (Willis, Rudner et al. 2000; Willis and van Andel 2004; Birks and Willis 2008). Climate modelling suggests that these areas would have been suitable for pine at that time (Cheddadi, Vendramin et al. 2006). Populations from the Iberian and Italian peninsulas harbour unique seed-transmitted mitochondrial DNA (mtDNA) variation that is not found elsewhere in Europe (Sinclair, Morman et al. 1999; Soranzo, Alía et al. 2000; Cheddadi, Vendramin et al. 2006; Pyhäjärvi, Salmela et al. 2008), and the Iberian pinewoods have also been found to differ from other continental populations for monoterpene and allozyme variation (Tobolski and Hanover 1971; Prus-Glowacki and Stephan 1994). These patterns support the view that more northern pine populations originate from refugia located north of the southern peninsulas and south of permafrost.

According to pollen studies, pine reached Scotland about 8,000 years ago and, appeared first in the Wester Ross area in the northwest, and then shortly afterwards in the Cairngorms (Birks 1989), the latter presumably having spread northwards through England (Bennett 1995). Interpreting pollen data in species like pine can be challenging due to its abundance and long dispersal distances, and therefore macrofossil data are needed to verify presence of local populations (Birks 2003). In fact, fossil stomata from two sites in the Highlands indicate that pine was locally present 1,600-600 years earlier than suggested by pollen data (Froyd 2005). Contemporary populations from Wester Ross differ from those in the rest of Scotland in their allozyme and monoterpene frequencies, suggesting that the contemporary Scottish population derives from multiple refugia (Forrest 1980; Forrest 1982; Kinloch, Westfall et al. 1986). For example, in contrast to the rest of the populations, the frequency of 3-carene in the northwest is very low (Forrest 1980); biochemically,

populations from this area seem more closely related to southern European populations than those from north-central Europe, which are similar to the rest of the Scottish pinewoods (Forrest 1982). It is possible that the north-western trees originate from refugia near southwest Ireland or western France (Ballantyne and Harris 1994; Bennett 1995), but this has not yet been verified by analysis of Irish macrofossils or potentially native pinewood remnants. Alternatively, natural selection or genetic drift may account for the differences, as these populations are on the edge of the species' range and under strong oceanic influence. The wet, mild climate is markedly different from that in other parts of the range and provides potentially divergent selective pressures involving, for example, pathogen attack, which may have driven biochemical differentiation. Biochemical similarity between northwest Scotland and southern Europe may reflect the effects of adaptation in a similar direction. However, if variation was due to drift, this would imply lack of gene flow between populations in western Scotland and elsewhere. In their mtDNA study, Sinclair, Morman et al. (1999) found two molecular variants in Scotland, the less common type being found in the western part of Scotland. Such differentiation further supports the view of colonization from two directions. Similarly, multiple origins might be suggested by the presence of a unique, paternally-inherited chloroplast DNA (cpDNA) microsatellite allele that was found only in the Wester Ross area (Provan, Soranzo et al. 1998). However, this variant could also represent a recent mutation. Had it been an ancestral polymorphism it would have been surprising that the allele was restricted to the area, considering efficient pollen-mediated transmission of cpDNA. Currently, the low number of mtDNA haplotypes detected prevents precise definition of the colonisation routes of pine in Europe (Sinclair, Morman et al. 1999; Naydenov, Senneville et al. 2007; Pyhäjärvi, Salmela et al. 2008), but further evidence for separate evolutionary origins of eastern and western pinewoods in Scotland has recently been found in candidate gene variation (Wachowiak, Salmela et al. 2011).

1.2.2 Current state of native pinewoods

During its history in Scotland, pine has fluctuated in abundance, sometimes very rapidly, due to various factors such as competition from deciduous tree species, decrease of deciduous forests, climate change, and human activity (Bennett 1995). Nowadays, the only natural pinewoods on the British Isles are patchily distributed in Scotland from latitude 55 ºN to 57 ºN and from longitude 3 ºW to 1 ºW at altitudes up to 600 metres (Mason, Hampson et al. 2004). According to the most recent available estimate, the native pinewood area in Scotland covers 18,000 hectares in 84 separate pinewoods varying in size from less than one to over 2,000 ha (Anonymous 1998); some populations are small and sparse consisting of little more than 100 trees at a density of less than one tree per hectare (e.g. Martin 1995). A substantial number of the native populations were already identified and described in the influential book 'The Native Pinewoods of Scotland' by Steven and Carlisle (1959). Natural pinewood regeneration is often prevented by grazing of domestic livestock or wild deer, muir burning, and planting of non-native trees (Anonymous 1998), and many of the populations have been reduced to very small numbers due to human interference. Also, in the past, trees of poor growth form have often been left in the forests while those considered to be superior from the silvicultural perspective have been felled and extracted for timber (Mason, Hampson et al. 2004). In such cases, the surviving trees could negatively affect the quality of later generations if they contribute to mating (Ennos, Worrell et al. 1998; Mason, Hampson et al. 2004). However, the extent of such practices is not known. In addition, undocumented quantities of trees of continental origin have been introduced to Scotland since the 19th century (Taylor 1993; Forrest and Fletcher 1995) which potentially could cause genetic contamination of local populations via pollen flow. The coverage of Scots pine plantations, which are mainly used for timber production, totals 100,000 ha (Mason, Hampson et al. 2004), but the extent to which they contribute to the pollen pool in Scotland is not known. Native trees of commercially desirable form persist in a few relatively large populations, e.g. Abernethy, Rothiemurchus and Glen Tanar (Mason, Hampson et al. 2004).

1.2.3 Management of genetic resources in Scots pine

Since the late 1980s, protection and expansion of pinewoods has been included in various policies and grant schemes (Mason, Hampson et al. 2004). For example, the 'Native Pinewood Grant Scheme' between 1989 and 2004 aided the regeneration of existing pinewoods and created 48,000 ha of new pinewoods (16% natural regeneration, 84% plantations) while the 'Native Pinewood Habitat Action Plan' aimed at increasing the remnant pinewood area by 5,600 ha by 2005 and assisting natural regeneration (McIntosh 2006). The Scottish government is aiming to increase forest land cover from 17.1 to 25% (Anonymous 2006), and also the commercial prospects for native pine are currently being re-evaluated, e.g. in 'Developing the Scots Pine Resource' project in collaboration with institutes from the Nordic Countries (Macdonald, Cooper et al. 2008).

However, despite the many unique characteristics of Scottish pinewoods, the extent of possible adaptive genetic differentiation among populations from environmentally diverse parts of Scotland has not been studied in detail. Scots pine is the only native tree in Britain to have its own, species-specific seed zones (Hubert and Cottrell 2007), and to guide seed transfers, the Scottish pinewoods have been divided into seven zones (figure 1.2) such that when (semi)-natural pinewoods are being expanded and in order to qualify for grant support, planting stock must come from within the same seed zone in an attempt to protect the local "genetic integrity" (Anonymous 1998). For other planting objectives, such as timber production, the rules are somewhat less restrictive. The seed zones are based largely on monoterpene studies (Forrest 1980) so that biochemically similar pinewoods are clustered within one zone. Although apparently practical where field data are in short supply, applying single-source data (such as monoterpenes and allozymes which can be considered selectively neutral molecular markers) to devise seed zones is likely, at best, to poorly reflect adaptive patterns (Merilä and Crnokrak 2001; McKay and Latta 2002) or, at worst, result in detrimental effects on survival and growth if environmental conditions vary greatly among the origin of seed and the plantation site.

Adaptive genetic variation among continental populations has been studied on many occasions in common-garden and reciprocal transplant trials, and environment-driven differentiation has been reported for instance in traits related to growth initiation in spring (Steiner 1979), growth cessation and the development of cold hardiness in autumn (Repo, Zhang et al. 2000). In Northern Sweden, long-lasting transfer trials in have shown that latitudinal transfers affect survival and growth (Eriksson, Andersson et al. 1980; Persson and Ståhl 1990). Hence, it appears that adaptation to local environments is common in Scots pine. However, the dimensions of environmental variation can vary spatially which means that results from an experiment carried out in one part of the range are not directly transferable to other areas. For instance, in Fennoscandia, many environmental factors such as growing season length and monthly temperatures vary along a latitudinal gradient (Savolainen 1996; Savolainen, Pyhäjärvi et al. 2007), and in Scots pine and other species, adaptive traits often have corresponding latitudinal clines (Hurme, Repo et al. 1997; Hall, Luquez et al. 2007). However, in spatially complex areas such as Scotland, geographical surrogates might not adequately explain variation in adaptive traits, and an understanding of how specific environmental factors vary across the landscape is called for.

1.2.4 Environmental variation within Scotland

Although the area covered by native Scots pine in Scotland is relatively small, environmental gradients within this area are steep (Mason, Hampson et al. 2004). To summarise climatic variation among Scottish native pinewood sites, Salmela, Cavers et al. (2010) extracted data for all 84 pinewoods from the gridded (5 × 5 km) long-term average (1961-1990) UK Met Office data (Perry and Hollis 2005). These data indicate that some western populations in Scotland experience an annual rainfall of close to 3,000 mm compared to only about 700 mm in more eastern parts of the country. The length of the growing season (the number of days with average temperature above +5°C) varies from about 100 in some eastern high-altitude

pinewoods to 300 days near the west coast. To study whether climatically similar sites were found within each seed zone, a principal component analysis (PCA) was performed to transform the seven variables into two components (figure 1.3, table 1.1). The data suggest that different pinewood sites within seed zones do not form climatically uniform clusters, which indicates that climatic variation within one zone can be large. For example, the South West seed zone covers areas with growing season lengths varying from about 180 to almost 300 days (table 1.2). In addition to climate, there is variation in soil types as well; generally pine prefers freely-draining podzol and ironpan soils with relatively low nutrient levels, but it is also found in brown earths, gleys, and peats (Mason, Hampson et al. 2004). In wet conditions, poor drainage can lead to poor growth and water-logging. Because of this extensive within-zone variation and considering for instance the effects of provenance transfers along latitudinal gradients in Sweden (Persson and Ståhl 1990), it is possible that current guidance results in seedlings being planted at non-optimal sites. It is essential for the maintenance of healthy pinewoods in Scotland that the patterns of adaptive variation in Scots pine across the country are investigated and taken into account when defining transfer guidelines for the species.
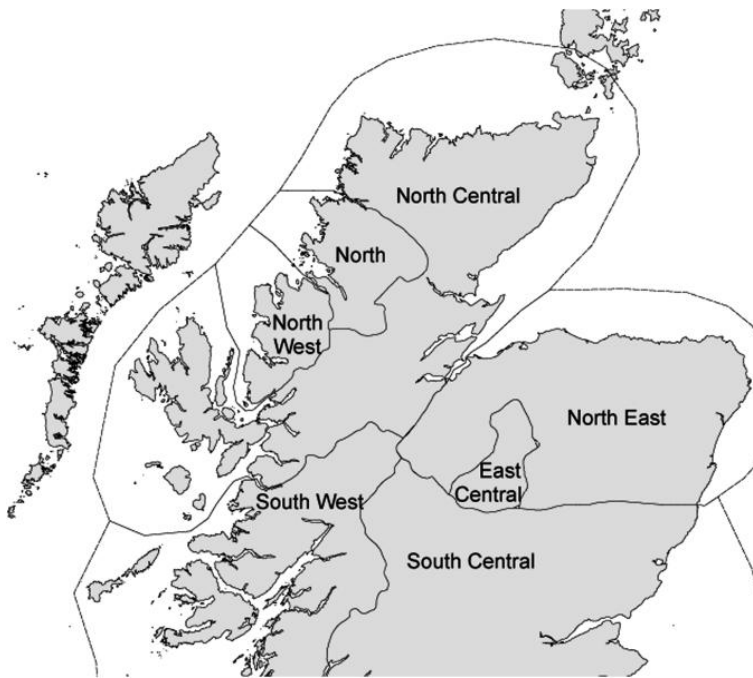
Figure 1.2 Map of the current Scots pine seed zones in Scotland.
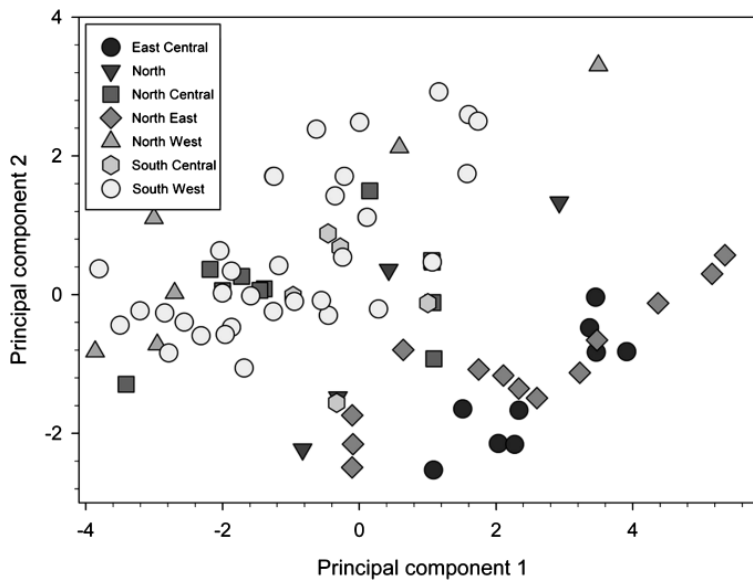
Figure 1.3 Plot of the two principal components (PC), which account for 69 and 24% of total variation, respectively, of climatic variation among 84 native pinewood sites. The seven variables used are shown in table 1.1. Current seed zones are represented by different symbols, and the closer the populations are in the graph, the more similar they are climatically. PC1 represents a gradient in annual rainfall and temperature: populations with more negative values are generally located in the west (high rainfall, mild climate); positive values represent more eastern pinewoods with less rainfall and colder winters.

Table 1.1 List of climatic variables used in the principal component (PC) analysis. Values in the table are correlation coefficients that vary between -1 (strong negative correlation) and 1 (strong positive correlation); the further the coefficient is from zero, the stronger the association between the variable and the PC. PC1 is the main component, explaining 69% of the variation.

Variable	PC1	PC2
Length of the growing season	-0.45	-0.12
February mean temperature	-0.45	-0.12
July mean temperature	-0.35	-0.47
Annual extreme temperature range	0.10	-0.72
Air frost days per year	0.44	-0.03
Ground frost days per year	0.43	-0.13
Annual precipitation	-0.30	0.46
Percentage of variation	69.2	23.99

Table 1.2 Range of climatic variation in four variables within each seed zone according to the UK Met Office long-term average data (Perry and Hollis 2005). Seed zones: EC=East Central, N=North, NC=North Central, NE=North East, NW=North West, SC=South Central, SW=South West. Climatic variables: LGS=length of the growing season, FMT=February mean temperature, JMT=July mean temperature, AP=annual precipitation.

Seed zone	LGS (days) min	LGS (days) max	FMT (°C) min	FMT (°C) max	JMT (°C) min	JMT (°C) max	AP (mm) min	AP (mm) max
EC	154	216	-0.8	1.0	10.6	13.0	743	1223
N	162	251	-0.5	2.4	10.0	13.9	1215	1778
NC	204	299	0.6	4.0	11.1	14.4	1346	2900
NE	108	234	-2.0	1.9	9.4	13.7	785	1343
NW	134	295	-0.9	4.0	8.5	14.0	1912	2790
SC	219	252	0.8	1.8	11.9	13.4	1159	2904
SW	179	297	0.0	3.9	9.7	14.1	1164	2934

1.2.5 Current knowledge about genetic variation in Scottish pinewoods

The current abundance of pinewood in Scotland is only a small fraction of what it used to be, and potentially the exploitation of the resources could have interfered with local adaptation by randomly removing best-adapted trees. However, the previous molecular marker studies based on monoterpenes (Forrest 1980; Forrest 1982) and allozymes (Kinloch, Westfall et al. 1986) and recent work on nucleotide variation in candidate genes (Wachowiak, Salmela et al. 2011) show that even in relict populations, levels of molecular variation are similar to those observed in the continuous part of the species' range and, as is usual in the case of long-lived, randomly mating forest trees with effective gene flow by pollen (Hamrick, Godt et al. 1992), almost all of the variation was found within populations. In theory, colonization events (such as postglacial migration) are expected to decrease genetic variation through bottlenecks, but the life-history characteristics of trees (longevity, multiple age and size classes, overlapping generations, and late reproduction) seem to buffer against these effects (Austerlitz, Mariette et al. 2000). For example, due to their postglacial colonisation history, northern Fennoscandian Scots pine populations are much more recently established than those from Central Europe (Willis, Bennett et al. 1998), but despite their different histories the two parts of the range have very similar levels of nucleotide variation at candidate genes (Pyhäjärvi, García-Gil et al. 2007). In Scottish populations, low marker divergence among populations suggests that gene flow among sites has, at least historically, been sufficient to homogenise genetic variation across populations (Kinloch, Westfall et al. 1986). Also, when comparing differentiation at cpDNA markers between Scotland and eight European mainland populations, only around 1.5 % of the variation was found between populations, indicating high levels of gene flow (Provan, Soranzo et al. 1998). Within Scotland, 3.2% of the variation was among populations. Glen Falloch, a relict population consisting of less than 100 trees, had the lowest diversity. Despite drastic changes in the abundance of Scots pine in Scotland, it seems that the level of neutral molecular variation remains high, with the majority of this variation being found mainly within populations.

Some evidence of local adaptation in the native pinewoods exists, but the data currently available are not extensive. Old provenance experiments set up by the Forestry Commission in Scotland starting in the 1920s show that populations from the mainland of Europe generally perform worse than Scottish material (Lines and Mitchell 1965; Worrell 1992). Within Scotland, trees transferred from continental to strongly oceanic areas usually perform worse than local populations, possibly due to pathogen stress (Mason, Hampson et al. 2004). Perks and McKay (1997) found significant differences in root frost hardiness and growth in seedlings from four provenances; for instance, seedlings from Loch Maree, located in the west close to the Atlantic, had poorer height growth and slower development of frost hardiness than other provenances. The only study where genetic parameters of adaptive variation were estimated was by Perks and Ennos (1999) who also sampled four provenances, each represented by 100 open-pollinated progeny (ten from each of ten mother trees). Seedlings were grown at one site and measured at seven years of age. Significant differentiation among populations was found in diameter, height, and bud burst. Adaptive variation was found in all of the measured characters, demonstrating the presence of genetic variation for adaptively important traits, but due to the sample size, estimates on the amount of adaptive variation are not precise. Also, while it was possible to show clear differentiation among populations in the traits considered, geographic coverage was too limited to offer a full picture of patterns of adaptive variation and the study did not attempt to link observed trends to variation in climatic variables.

1.2.6 Maintenance of adaptive potential in native pinewoods

Ongoing climate change is affecting forests all over the world, and changes in temperature, rainfall, and frequency of extreme weather events are expected (e.g. IPCC 2007). In Scotland, models predict warmer summers and milder winters, with changes in the distribution of rainfall (Ray 2008). In the east, summers are predicted to become drier, possibly leading to drought, while winters may become wetter, also a problem if it leads to water-logging and anaerobic conditions in soils. Warmer

conditions may help pests and pathogens spread to new areas. For example, the northward spread of the pine processionary moth (*Thaumetopoea pityocampa* Dennis and Schiff) in Italy has been attributed to increasing winter temperatures (Battisti, Stastny et al. 2005), and since the late 1990s, the occurrence of red needle blight, a fungal disease infecting a wide range of *Pinus* species, has increased in the UK with first outbreaks occurring in Scotland in 2002 (Brown, Rose et al. 2003). Such changes can lead to situations where environments are no longer optimal for the populations growing in them. Trees have experienced warming conditions before, following the retreat of continental ice at the end of the ice age (e.g. Davis and Shaw 2001). In current conditions the problem for trees is likely to be the rate of change which is projected to be faster than that following the latest ice age. After the last glaciation, European trees migrated at average speeds of around 100-700 metres per year, depending on the species (Brewer, Cheddadi et al. 2002; Magri, Vendramin et al. 2006). According to Malcolm and Markham (2002), trees will have to be able to migrate at a rate of over 1,000 m per year to be able to keep pace with human-induced change. This time, however, trees face environments already occupied by other species.

For a change in fitness of the population, selection must work on the variation present in the population (Falconer and Mackay 1996). Only variation that can be passed on to the next generation is of evolutionary importance and therefore, to estimate whether a trait is genetically inherited and whether variation is found within populations, a progeny trial consisting of families of a known structure (e.g. half-sibs) is needed. In forest trees, the most common approach is to collect open-pollinated seed from many mother trees and to assume that such progeny are mostly half-sibs (e.g. White, Adams et al. 2007). When many populations and families within populations are sampled, total phenotypic variation observed in a common-garden trial can then be divided into among- and within-population (among-family) components. Quantitative genetic models can then be used to calculate additive genetic variance V_A and narrow-sense heritability which is defined as the proportion of total phenotypic variation due to additive gene effects ($h^2=V_A/V_P$; Falconer and Mackay 1996). For example, when examining true half-sib families, V_A is estimated

as $4 \times$ among-family variance component. In the majority of the forest trees studied, high levels of within-population variation in adaptive traits have been documented (Howe, Aitken et al. 2003; Savolainen, Pyhäjärvi et al. 2007; Aitken, Yeaman et al. 2008), even in range-edge populations under extreme conditions (Savolainen, Bokma et al. 2004; Notivol, García-Gil et al. 2007). The evolution of native pinewoods will depend on adaptive variation within populations, and it is important that experimental designs will allow estimating within-population diversities, too.

1.2.7 Combining quantitative trait and molecular marker data

Studies on adaptive variation would also benefit from an understanding of current patterns of genetic connectivity among forest fragments. For example, if only local material is used for planting and gene flow is limited, local genetic "integrity" of small populations will be maintained, but the population might become vulnerable to changing conditions due to insufficient adaptive variation for natural selection to operate on. In the case of isolated populations, variation could be introduced by bringing seedlings from other locations. However, if gene flow occurs naturally and if natural regeneration occurs, such practices might be unnecessary.

Due to differences in the sizes of the native pinewoods (from less than one to over 2,000 ha), there might also be variation in the patterns of mating system. In small populations, random drift becomes a powerful force shaping allele frequencies, and along with inbreeding, this can lead to lower fitness as detrimental alleles increase in frequency (Frankham, Ballou et al. 2002). Like other pines, Scots pine is mainly outcrossing (Muona and Harju 1989), i.e. matings usually occur between unrelated trees, but self-pollination, the most severe form of inbreeding, is also possible due to the lack of a genetic system preventing self-fertilization (Sarvas 1962). Normally, selfed embryos are aborted early in their development due to early inbreeding depression. However, in stands with limited numbers of trees, bi-parental inbreeding (mating between relatives) is a potential risk. Despite efficient gene flow, inbreeding might become a significant factor when isolation is extreme. In Scots pine, gene flow

and mating system have been studied for instance in Spanish populations occurring in isolated stands in mountainous regions. Although the proportions of self-pollination were eight times larger (25% vs. 3%) in a population of 36 trees spread across a 15-ha area compared to that of larger populations covering thousands of hectares (Robledo-Arnuncio, Alía et al. 2004), the rates were nevertheless low when the degree of isolation of the trees is taken into account. In the small population, 4.3 % of the pollen originated from other populations, the closest one being located 30 km away (Robledo-Arnuncio and Gil 2005). Kärkkäinen, Koski et al. (1996) documented variation in levels of inbreeding depression within larger populations in Finland: outcrossing rates in northern populations were somewhat lower than in the south, but inbreeding depression was weaker in the north, possibly due to selection having already removed detrimental recessive alleles exposed by inbreeding. Understanding the mating system is also beneficial for studies on adaptive variation in phenotype, as departures from the assumed family structure can lead to biased estimates of adaptively significant genetic variation (Namkoong 1966; Squillace 1974). If half-sib families also contain full-sibs, estimates of V_A can be biased upwards. A common approach has been to use a multiplier smaller than four to account for some full-sibs among progeny (e.g. Campbell, Pawuk et al. 1989).

1.3 The objectives of this thesis

Scots pine is one of the most intensively studied forest trees in the world, but only little is currently known about the patterns of adaptive trait variation in Scots pine in Scotland. Environment-driven genetic differentiation among continental European populations has been demonstrated in other studies, and considering the extensive spatial environmental heterogeneity found among Scottish pinewoods, it is possible that adaptation to local environments has also occurred in Scotland and that current seed transfer guidelines that are based on molecular markers allow transfers too far along environmental gradients. In order to explore whether adaptive genetic differentiation among Scottish pinewoods has taken place and to maximise the success of future replanting efforts, a range-wide, family-structured common-garden experiment is needed.

The purpose of this thesis is to investigate the evolution of Scots pine in Scotland by examining associations between environmental variables and patterns of phenotypic variation observed in a common-garden trial consisting of 84 open-pollinated families from 21 native pinewoods. This approach will allow the division of phenotypic variation in a large number of seedlings into among- and within-population components and testing for relationships between trait means and environmental characteristics of each population and family's home site. The following questions will be addressed:

1. Does the level of outbreeding vary among populations? This question is examined in Chapter 2 by genotyping seedlings from all studied families at polymorphic microsatellite markers. Understanding mating system variation is important for interpreting patterns of quantitative trait variation in the subsequent experiments.

2. Does timing of bud flush vary among populations occupying sites with contrasting annual temperature features? This question is addressed in Chapter 3 in which data from two years and two separate common-garden

trials are analyzed. In addition, the potential role of temporally varying climate in maintaining quantitative trait variation within populations will be discussed.

3. Do populations from sites with contrasting annual rainfall differ in their response to droughting and are possible differences related to their home site conditions? In Chapter 4, seedlings from five populations were subjected to drought and chlorophyll fluorescence along with measurements of water deficit in needles and mortality were used to assess their response.

4. Do populations from sites experiencing different annual temperature regimes vary in response to natural winter temperatures and are the observed patterns associated with the home environments of the populations? In Chapter 5, data are presented from an outdoor experiment in which chlorophyll fluorescence between autumn and spring and spring phenology were monitored in seedlings from eight populations.

Chapter 6 will summarise the results from these experiments and propose future research needed for a better understanding of native pinewood biology in Scotland. In addition, policy recommendations concerning the seed sourcing of Scots pine in Scotland will be made.

2. Mating system in Scots pine (*Pinus sylvestris* L.) in Scotland

2.1 Introduction

The distribution of genetic variation among and within populations is one of the key questions in evolutionary biology, an understanding of which is needed for e.g. defining units of conservation in natural populations (Frankham, Ballou et al. 2002). A major component that determines the distribution of genotypes in a population is the mating system, i.e., the relative amounts of outcrossing and inbreeding (Mitton 1992), while gene flow contributes to the distribution of variation among populations (Slatkin 1987). Inbreeding (selfing or mating among relatives) decreases genetic diversity and increases structuring of diversity within and among populations, while outcrossing promotes homogeneity among them (Loveless and Hamrick 1984).

Forest trees generally have a mixed mating system, with some proportion of seed (s) being produced through self-pollination and the rest via outcrossing ($t=1-s$) (Mitton 1992). Patterns among species show distinct variation, but in general, trees are highly outcrossing which is also seen in the high amount of genetic diversity within populations and in low levels of differentiation among even geographically distant populations (Hamrick, Godt et al. 1992; White, Adams et al. 2007). Factors that promote very high levels of diversity in trees are, e.g., large population sizes, longevity and efficient wind-mediated gene flow via pollen (Ledig 1998). However, although high diversities are often found in both molecular markers and quantitative traits, the latter often show significant population differences in response to the environmental conditions at the home site of the population, showing that selection can counteract the homogenizing effect of gene flow (e.g. Howe, Aitken et al. 2003).

Studies on genetic structures among adult trees in natural populations have often shown no or only very weak signs of inbreeding (e.g. Yazdani, Muona et al. 1985; Kärkkäinen, Koski et al. 1996). However, a different pattern emerges when analyses

focus on seed collected in mother trees. Such studies have shown that selfing actually accounts for a significant proportion of mating events (Sarvas 1962). In terms of molecular markers, this is seen as an excess of homozygotes (Charlesworth and Charlesworth 1987). The difference between the levels of homozygosity at different life stages can be explained by the high early inbreeding depression that efficiently removes highly inbred individuals from progeny (Williams and Savolainen 1996). In outcrossing species, inbreeding generally results in reduced performance and fitness compared to outcrossed progeny (Charlesworth and Charlesworth 1987), and similar effects have also been observed in trees (Sorensen and Miles 1974; Williams and Savolainen 1996). However, high outcrossing does not exclude the possibility of mating between relatives. In small and fragmented populations outcrossing rates can still be high due to pollen flow from other sites (Smouse and Sork 2004), but there might be differences in the number of pollen donors and in the proportion of full-sibs in progeny among small and large stands (O'Connell, Mosseler et al. 2006). These observations have been made by studying seed from small and large populations with varying tree density. Less frequently, the mating system has been assessed in seedlings or adult trees (e.g. Sorensen and White 1988; Gaspar, de-Lucas et al. 2009). Although inbreeding depression can remove highly inbred individuals before they become established as seedlings, in controlled experiments their chances of survival could be increased and some among-family or population variance due to inbreeding might be interpreted as genetic differences. When estimating parameters of variation in quantitative traits, progeny are often presumed to be half-sibs, but if full-sibs are also present, estimates of quantitative variation can be erroneous (Namkoong 1966; Squillace 1974).

The aim of this study was to examine 1) whether mating system varied among 21 populations and 84 open-pollinated Scots pine families in Scotland, and 2) whether such variation could contribute to the patterns of quantitative trait variation (first and second year growth and variability in the same characters) within and among the same population and families.

2.2 Materials and methods

2.2.1 DNA extraction

1,680 seedlings from 84 open-pollinated families and 21 populations were sampled for the study. DNA was extracted from current-year needles sampled in November and December 2008 under common-garden glasshouse conditions, and approximately 50 mg of needle material was taken per plant. Needles were cut into smaller pieces while still fresh and then stored in a freezer at -20°C prior to extraction.

DNA was extracted using the QIAGEN DNeasy 96 Plant Kit (QIAGEN) protocol with the following modifications: 1) needle grinding was performed twice for 30 s at 30 Hz; 2) in procedures where centrifuging at 6,000 rpm was required, the centrifuge used was set to $1.5 \times$ the time given in the handbook; 3) final elution was to 100 μl (first round) and to 50 μl (second round). In some cases needle grinding was performed the day before the extraction, in which case the lysed samples were stored at -80°C overnight.

Extractions from the first elution (1 μl sample + 3 μl dye) were run on a 1% agarose gel to check the amount of DNA. Following the extractions, all extracts were stored at -20 °C.

2.2.2 Polymerase-chain reaction (PCR)

Five microsatellite loci were used in the study: SPAC11.8, SPAG7.14 (Soranzo, Provan et al. 1998), PtTX3017, PtTX4001, and PtTX4011 (Auckland, Bui et al. 2002). The first two microsatellites were developed from Scots pine total genomic libraries, while the remainder were developed from different DNA libraries of loblolly pine (*P. taeda* L.). The PCR reagent mix and programme protocols used for

these markers are shown in table 2.1 and 2.2, respectively. All reactions were performed on MBS Satellite 0.2G Therma Cycler (Thermo Hybaid).

Table 2.1 PCR reagent mix protocols (per one sample) used for the five primers.

1 sample (µl)	H_2O	10 × buffer	Primers F/R (5 µM)	dNTP	*Taq*	MgCl (25 µM)	DNA	BSA
SPAC11.8	7.7	1.5	0.75	0.6	0.2	1.2	2[a]	0.3
PtTX3107	8.9	1.5	0.75	0.6	0.2	1.5	0.5[a]	0.3
SPAG7.14	7.7	1.5	0.75	0.6	0.2	1.2	2[b]	0.3
PtTX4001	7.4	1.5	0.75	0.6	0.2	1.5	2[b]	0.3
PtTX4011	7.4	1.5	0.75	0.6	0.2	1.5	2[b]	0.3

[a]=DNA extraction dilution 1/20

[b]=DNA extraction dilution 1/5

Table 2.2 PCR programme protocols used for a) SPAG7.14, b) PtTX4001 and PtTX4011, and c) SPAC11.8 and PtTX3107.

a) SPAG.7.14

Cycles	Treatment	T (°C)	Time
1	Denaturation	94	3 min
10	Denaturation	94	30 s
	Annealing	61 (-0.7°C/cycle)	30 s
	Extension	72	30 s
35	Denaturation	94	30 s
	Annealing	54	30 s
	Extension	72	30 s
1	Final extension	72	5 min
	Hold	4	

b) PtTX4001/PtTX4011

Cycles	Treatment	T (°C)	Time
1	Denaturation	94	2 min
20	Denaturation	94	30 s
	Annealing	60 (-0.5°C/cycle)	30 s
	Extension	72	30 s
15	Denaturation	92	30 s
	Annealing	50	30 s
	Extension	72	1 min
1	Final extension	72	15 min
	Hold	4	

c) SPAC11.8/PtTX3107

Cycles	Treatment	T (°C)	Time
1	Denaturation	94	4 min
35	Denaturation	94	45 s
	Annealing	57	45 s
	Extension	72	45 s
1	Final extension	72	5 min
	Hold	4	

2.2.3 Agarose electrophoresis

To check for successful amplification, a small set of samples from each PCR plate was visualised on a 1.5% agarose gel run at 100V for 30 min-1 hour. 1 μl of PCR product was added to 3 μl of loading buffer.

2.2.4 Genotyping

Microsatellite alleles were resolved on 6% polyacrylamide gels using a Long ReadIR 4200 sequencer (Li-Cor). Gels were run for 1.5-2 hours, and genotyping was done using SAGA Generation 2 software. All samples were checked visually, and after initial genotyping, the results were grouped into families.

2.2.5 Mating system analysis

Mating system in populations and families was examined using MLTR ver. 3.2 (Ritland 2002). The programme is based on a mixed mating model, assuming that a plant's progeny results from both self-fertilization and outcrossing. Estimated parameters were multi-locus (t_m) and single-locus (t_s) outcrossing rates and correlated paternity within maternal families (r_p). The difference between t_m and t_s reflects matings between relatives (bi-parental inbreeding). Standard deviations were estimated by running the programme with 500 bootstraps and the EM algorithm (for population estimates, families within populations were resampled, and for family-level estimates, individuals within families were resampled). Population t_m and r_p estimates were compared to the latitude, longitude and altitude at origin, and also to the core pinewood size according to Mason, Hampson et al. (2004), means of first and second year growth, and population coefficients of variation (CV) in the same growth traits. For the family-level analyses, two additional variables measured during seed collection were added to the analysis: 1) height of the mother tree, and 2)

average distance to the three closest trees. Ratios were transformed prior to use in regression analyses by $X/(1-X)$, where X=t_m or r_p.

2.2.6 Growth characters

The height of all seedlings in the common-garden trial (~3,360 seedlings) was measured in February 2008. This was considered as the first year height. Second year growth was defined as the difference between the measurements taken in December and February 2008. Population and family means, along with corresponding coefficients of variation (*CV*), were calculated for these traits.

2.3 Results

2.3.1 Microsatellite amplification

26 alleles were scored for SPAC11.8 (120-178 bp; 1,183 samples), 8 for PtTX3107 (151-172 bp; 1,202 samples), 33 for SPAG7.14 (179-239 bp; 1,157 samples), 15 for PtTX4001 (198-230 bp; 1,185 samples), and 8 for PtTX4011 (256-278 bp; 1,134 samples).

2.3.2 Mating system analysis

Prior to analyses, individual genotypes within each family were visually checked to see if the maternal genotype could be inferred from the data. Alleles of PtTX4001 and PtTX4011 were consistent with one family sharing a common parent (except for one family at PtTX4001); at SPAG7.14, progeny genotypes were not compatible

with the assumption of one common parent in 31 families and at PtTX3107 in 45 families. At SPAC11.8, genotypes in only 13 families were compatible with each family sharing one common parent. Maternal samples were not genotyped separately, and for this reason mating system analysis was carried out using only PtTX4001, PtTX4011 and SPAG7.14.

Outcrossing rates (t_m) among populations varied between 0.815 ($\pm$ 0.054) in Allt Cul to 0.951 ($\pm$0.011) in Glen Cannich when using two loci (PtTX4001, PtTX4011), and from 0.794 (+0.08) in Coille Coire Chuilc to 0.976 ($\pm$0.006) in Glen Cannich when adding SPAG7.14. The lowest estimates were associated with highest standard deviations (for three loci, $r=-0.6385$, $p<0.0018$). Population t_m estimates between the two- and three-locus sets were significantly correlated ($r=0.7786$, $p<0.001$), and the correlation between two and three-loci r_p was marginally significant ($r=0.4750$, $p=0.0296$). In the following analyses only three-locus data are used. Estimates of bi-parental inbreeding (t_m-t_s) varied between -0.059 ($\pm$0.045) in Coille Coire Chuilc to 0.0840 ($\pm$0.0230) in Shieldaig, and estimates of correlated paternity (r_p) were found to vary between 0.0530 ($\pm$0.009) in Rothiemurchus and 0.10 ($\pm$0.009) in Coille Coire Chuilc. Population estimates of t_m, t_m-t_s and r_p are shown in figures 2.1, 2.2, and 2.3, respectively.

Population t_m's were not associated with latitude, longitude, altitude, or pinewood size ($p=0.313$, $p=0.656$, $p=0.954$, $p=0.640$, respectively). No associations were found between t_m's and mean first year growth ($p=0.304$), mean second year growth ($p=0.377$), first year growth CV ($p=0.483$) or second year growth CV ($p=0.801$). Similar results were obtained with r_p, and none of the associations were statistically significant.

On the family level, t_m's and r_p's were compared to the same growth variables. Family t_m's were not associated with mean first year ($p=0.251$) or second year growth ($p=0.898$), or first ($p=0.654$) or second year growth CV ($p=0.949$). Similar results were obtained when r_p was used as the explanatory variable. Average distance

to three closest trees was not associated with either t_m ($p=0.291$) or r_p ($p=0.317$), and neither was the height of the mother tree (for t_m, $p=0.196$; for r_p, $p=0.860$).
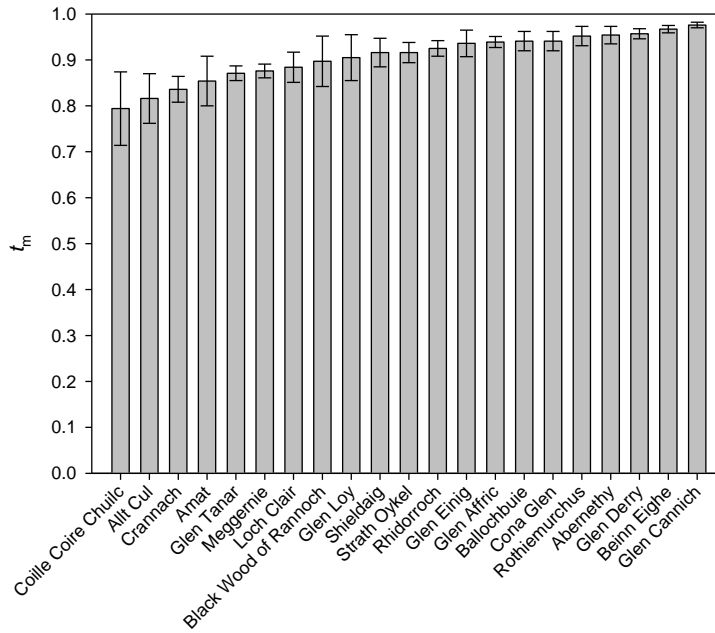
Figure 2.1 Population estimates of t_m. Error bars mark 95% confidence intervals.
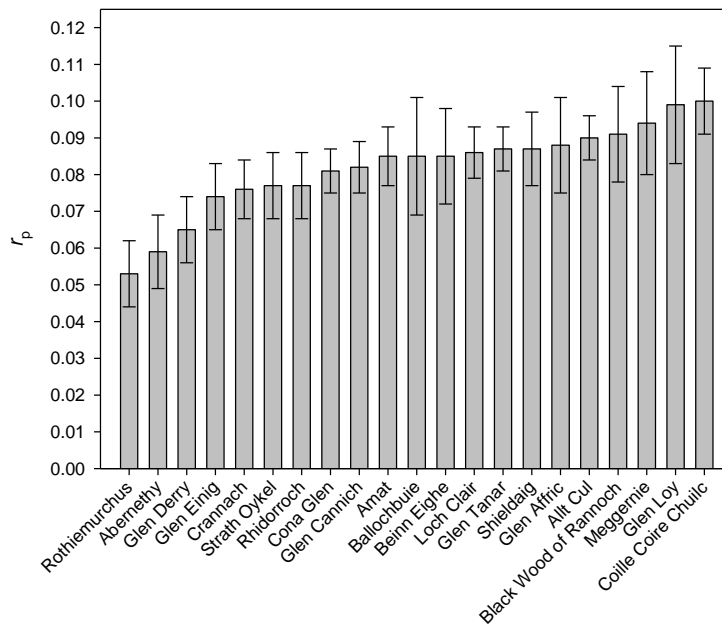
Figure 2.2 Population estimates of r_p. Error bars mark 95% confidence intervals.
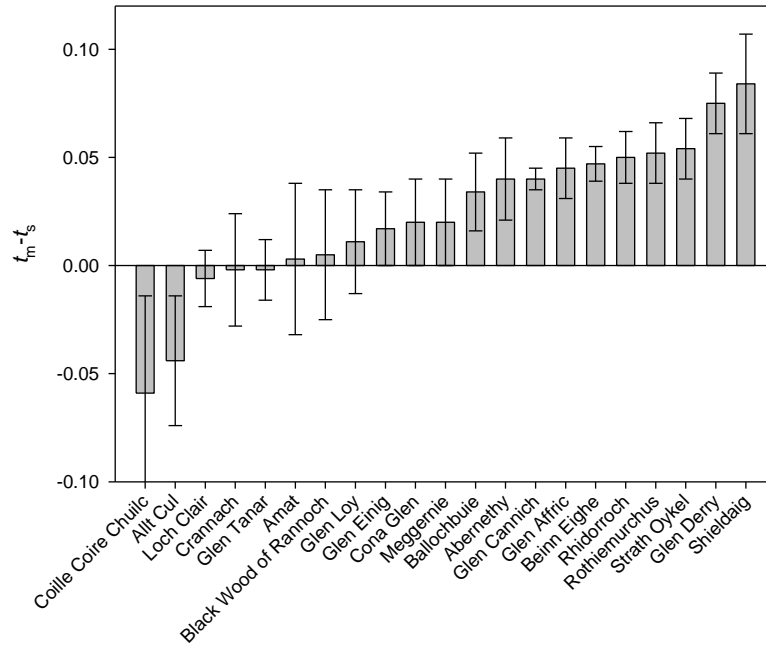
Figure 2.3 Population estimates of t_m-t_s. Error bars mark 95% confidence intervals.

2.4 Discussion

This study examined mating system variation among 21 native Scots pine populations in Scotland by genotyping ~1,200 progeny from 84 open-pollinated families at five microsatellite loci. Analyses were done using only three loci at which progeny genotypes were compatible with a family sharing one common parent. Despite a limited number of loci, the results show that outcrossing rates across Scotland are high, although the range of variation in population t_m's (from 0.794 to 0.976) is more extensive than in other studies on the same species. Estimates of correlated paternity varied from 0.053 to 0.1, and evidence of bi-parental inbreeding was found in 10 populations.

2.4.1 Microsatellite amplification

Only three loci (two in some families and populations) were used in the mating system analysis as at the other two loci, most progeny genotypes could not have come from a family that shared at least one common parent. At the highly-variable SPAG7.14, this pattern was found in 31 families. The problem was due to an excess number of different homozygote genotypes within families which might be caused by the presence of null alleles or by the unequal amplification of the two alleles during PCR. In previous Scots pine studies embryos along with haploid maternal tissues in seed have been simultaneously genotyped which can help in determining the cause of non-matching genotypes. To improve this dataset, additional highly-variable loci which do not have null alleles should be added. While markers developed for loblolly pine are often transferable to Scots pine, they should first be tested on a small number of families to determine whether they work reliably. Besides the markers developed by Soranzo, Provan et al. (1998), no Scots pine-specific markers are yet available. Paternally-inherited chloroplast DNA markers have also been used in previous Scots pine studies (Robledo-Arnuncio, Alía et al. 2004; Robledo-Arnuncio and Gil 2005).

2.4.2 Variation in mating system

Although only a very small number of loci was used in this mating system analysis, the results show high levels of outcrossing in Scottish pine populations. Population estimates varied from 0.794 to 0.976, but the lowest estimates were also associated with largest confidence intervals. These patterns are in accordance with previous studies on mating system in Scots pine and other *Pinus* species (Williams 2009), although the values are smaller in some populations than those reported in other Scots pine studies. This study differs from most other mating system studies in that it sampled two-year old seedlings growing in a common-garden experiment, while in other Scots pine studies seeds have been sampled (Muona and Harju 1989; Robledo-Arnuncio, Smouse et al. 2004; Robledo-Arnuncio, Alía et al. 2004). Because of this,

one would expect to see at least similar or even higher t_m's in the data from this experiment (see below), and it is likely that the lower t_m's observed here are at least partially due to the limited set of loci used.

Nearly all conifers studied so far have been found to be predominantly outcrossing and only a few examples of t_m being lower than 0.55 have been reported (Williams 2009). Muona and Harju (1989) reported t_m of 0.95 in Finnish Scots pine seed orchards, and t_m's were very similar among four Spanish Scots pine stands that have experienced different silvicultural practises (Robledo-Arnuncio, Smouse et al. 2004). In these data, t_m is significantly different among some populations, but as only four families within each population and 13-16 progeny/family were genotyped, these data may be less reliable than those of previous studies in which within-population sampling was more intensive. Extensive variation in outcrossing rates can be found among individual trees within populations (e.g. Kärkkäinen, Koski et al. 1996; de-Lucas, Robledo-Arnuncio et al. 2008), a pattern that could contribute to the observed seedling-stage differences in t_m among populations. Ritland (2002) has suggested that ~400 progeny/population are likely to yield good estimates, but the number can be decreased if highly polymorphic loci are used.

Despite being highly outcrossing, pines are capable of selfing as they do not have a self-incompatibility system, and selfing rates of 10-25% have been reported for Scots pine in large normal density populations, with the rate increasing towards lower densities (Sarvas 1962). The possibility of selfing can be decreased by spatial separation of male and female flowers, differences in timing of male and female flowering, and differential investment in male and female flowers (Williams 2009). For example, in Scots pine, a negative correlation between pollen and cone production has been reported (Savolainen, Kärkkäinen et al. 1993), and female flowers can become receptive before pollen from the same tree starts to be produced (Koski and Tallqvist 1978). Also, long-distance pollen flow is very efficient, and a large number of males usually contribute to the pollen pool sampled by one mother tree (Smouse and Sork 2004). For example, the effective number of pollen donors among four Scots pine stands in Spain has been estimated to vary between 71 and

125 (Robledo-Arnuncio, Smouse et al. 2004). Strong pollen-mediated gene flow is also likely to contribute to the low among-population differentiation that has been documented at allozyme, monoterpene (Kinloch, Westfall et al. 1986), and cpDNA markers (Provan, Soranzo et al. 1998) in Scots pine in Scotland. Early inbreeding depression in pines is severe, meaning that selfed progeny are efficiently aborted very early in their development, most likely due to the expression of embryonic lethal genes (Sarvas 1962). Koski (1971) has estimated that in controlled selfing in Scots pine, ~85% of the ovules do not produce live seed. Also, the germination and growth of inbred individuals are often poorer compared to outcrossed progeny, further decreasing their chance of survival, especially under natural conditions (e.g. Ledig 1998). This selective process explains why excess homozygosity observed in embryos often disappears by the adult stage (Yazdani, Muona et al. 1985). The estimates of t_m and r_p were compared to the growth characteristics of the populations and families, and found no significant associations, suggesting either 1) poor resolution due to the small marker data set, or 2) lack of significant differences among populations or families. Family-level estimates are difficult to obtain accurately, and it has been suggested that estimates on that level should be examined jointly with factors that might cause variation among families such as density or size of the mother plant (Ivey and Wyatt 1999; Ritland 2002). In maritime pine (*Pinus pinaster* Aiton), positive but weak ($R^2<15\%$) associations between family t_m's and mother tree and crown size have been found (de-Lucas, Robledo-Arnuncio et al. 2008). These associations could be expected to become significantly weaker if analyses were based on seedlings or adult trees.

Although outcrossing rates in pines are often high and above 0.9, differences between the single-locus and multi-locus estimates are frequently observed, indicating bi-parental inbreeding, i.e. breeding between relatives, which depresses t_s while having less effect on t_m (Ritland 2002). This could be due to the spatial structuring of related individuals in a population (Ledig 1998). In this study, t_m-t_s's were different from 0 in 10 populations, but among those populations the estimates were very similar, suggesting mating between relatives at a rate of approximately 5%. Confidence intervals increased towards higher estimates. In a similar study,

Gaspar, de-Lucas et al. (2009) genotyped five families in a breeding trial of ~20-year old maritime pine to examine whether the assumption that all families consist of half-sibs was violated. They found evidence of correlated paternity among maternal progeny, but the proportion of full-sibs was relatively low and had little effect on the estimates of heritability. No associations were found between outcrossing variables and patterns of variation in wood quality traits. Based on these observations, it seems likely that the progeny in this trial are outcrossed, although more markers need to be added to the analysis before this finding can be confirmed (smaller confidence intervals of t_m and r_p).

Although analyses of seedlings or adult trees might be unable to show differences in realized outcrossing among groups of seedlings, variation can occur among populations that vary, e.g., in density and degree of isolation when estimates are based on genetic structures in seed. In whitebark pine (*Pinus albicaulis* Engelm.), populations in southern British Columbia were found to have clearly lower t_m than populations from Oregon or Montana (Bower and Aitken 2007), and in Sitka spruce, higher selfing and correlated paternity were found in peripheral and isolated northern and southern populations compared to more continuous central populations (Mimura and Aitken 2007). In Scots pine in Finland, slightly lower t_m (0.93) was discovered in northern populations compared to those in the southern parts of the country (0.99), and early inbreeding depression was less severe in the north (Kärkkäinen, Koski et al. 1996). However, at the seedling and adult stages the proportion of inbred individuals did not vary among populations. It is possible that some populations can tolerate higher levels of inbreeding due to a smaller number of lethal genes which have been exposed by selfing in the past and subsequently purged by natural selection (Kärkkäinen, Koski et al. 1996). In peripheral populations the possibility of self-pollination can be increased by lower tree densities and lower levels of pollen production. Outcrossing rates could also vary among Scottish populations if there is variation in tree density and in pollen production due to temporal fluctuation in environmental conditions. However, it has been suggested that in trees, population size decrease has to be very severe for it to have an effect on the mating system (Robledo-Arnuncio, Alía et al. 2004). The size of the pinewoods studied here varies

from 13 to over 2,000 ha (Mason, Hampson et al. 2004), and it is possible that at this scale no major differences occur between populations if tree densities remain sufficiently high (above 20 trees/ha based on Robledo-Arnuncio, Alía et al. 2004). Selfing rates could be expected to be high in geographically isolated and highly fragmented pinewoods, e.g. in Glen Falloch, which consists of less than 100 trees (Martin 1995). In Spain, an isolated pinewood with 36 trees at a density of 2.4 trees/ha had an eight times larger selfing rate and 100 times bigger correlated paternity compared to large populations with densities above 80 trees/ha (Robledo-Arnuncio, Alía et al. 2004).

2.5 Conclusions

Considering the early-acting and intense selection against highly inbred individuals in pines and the results from this rather limited study, it seems likely that the progeny in this common-garden trial are outcrossed and that possible population differences in the level of outcrossing are small. However, this does not exclude the possibility of mating between related individuals or selfing at earlier developmental stages of seed. For better estimates of t_m and r_p and also for estimating the (realized) genetic structure of pollen clouds sampled by different mothers across Scotland, the following is needed:

1. More highly polymorphic markers. Currently only a limited number of Scots pine-specific nuclear markers are available (Soranzo, Provan et al. 1998), although resources are much more extensive for loblolly pine (Auckland, Bui et al. 2002). These markers can be transferred between species, but it recommended that tests are first carried out on a small set of families to test whether those markers can be used for determining the maternal genotype.
2. In case of problematic loci (e.g. excess homozygosity in progeny), the inclusion of maternal samples in the genotyping array could help.

3. For estimates of selfing rate and differences in inbreeding among populations, seed should be sampled and more intensive within-population sampling would be required.

3. Variation in timing of bud flush among native pinewoods in Scotland

3.1 Introduction

Genetic differentiation among populations due to spatial environmental heterogeneity is a commonly observed pattern in many quantitative traits of plants (Linhart and Grant 1996). Studying genetic variation in adaptive traits of forest trees from the northern hemisphere has a long history due to the commercial importance of many species, and many studies have reported significant divergence among populations from ecologically diverse conditions when grown in common-garden conditions (e.g. Howe, Aitken et al. 2003). In many northern areas, the length of the growing season varies along latitudinal or altitudinal gradients, and in order to avoid late frost in spring and early frost in autumn, trees which time their active growth so that it overlaps with the warm period at their home site have a selective advantage. This allows them to develop hardiness before potentially damaging cold temperatures in autumn. Such latitudinal patterns in for example timing of growth cessation and cold hardiness are often documented in tree populations from Fennoscandia (reviewed in Savolainen, Pyhäjärvi et al. 2007). In species occurring in more spatially complex areas such as the Pacific Northwest, the patterns of variation can be more complicated and trait variation might not be linearly associated with geographic surrogates such as longitude or latitude. For example, Sorensen (1983) studied adaptive variation among Douglas-fir populations from coastal and inland ridges (east and west aspects) in Oregon and found larger phenological differences among the two aspects of the coastal ridge than among those on the inland ridge. Furthermore, the direction of elevation trends varied between the two aspects of the coastal ridge. Later analyses on adaptive variation in the same species have been able to use fine-scale climate data to characterise the environments occupied by populations, and patterns of variation for instance in traits related to timing of growth

have been found to be related to winter temperatures and summer drought (St. Clair, Mandel et al. 2005).

In addition to studying how means in quantitative traits vary among populations from diverse environments when growing under common-garden conditions, the amount of molecular marker diversity has also been extensively investigated in trees. Selectively neutral markers and quantitative traits often show very contrasting patterns of variation: in terms of genetic markers, the great majority of the variation resides within populations, while for quantitative traits, a greater proportion of the variation is accounted for by among-population differences (e.g. Karhu, Hurme et al. 1996; Yang, Yeh et al. 1996). A common observation is also that populations from different parts of the range have very similar levels of marker diversity within populations (e.g. Kinloch, Westfall et al. 1986; Gapare, Aitken et al. 2005; Pyhäjärvi, García-Gil et al. 2007). Similarly, high levels of within-population variation are also often seen in quantitative traits (Howe, Aitken et al. 2003), but possible differences in the amount of quantitative trait variation among multiple populations have not been extensively studied. Studies on southern and northern Finnish Scots pine have revealed high levels of quantitative variation in timing of bud set, cold hardiness (Savolainen, Bokma et al. 2004), and pollen and cone production (Savolainen, Kärkkäinen et al. 1993) in both parts of the country. This has been considered surprising as the sampled northern areas are close to the range limit of Scots pine where strong directional selection has been assumed to occur (Savolainen 1996). Spatial heterogeneity and extensive pollen-mediated gene flow are expected to be major determinants of the level of variation in adaptive traits (Howe, Aitken et al. 2003). However, although it has been suggested that a temporally varying environment (and selection) might influence the levels of adaptive variation within tree populations (Howe, Aitken et al. 2003; Westfall and Millar 2004; Yeaman and Jarvis 2006), no studies have yet explored this possibility in more detail by analysing patterns of both temporal climate and quantitative trait variation.

The 84 native Scots pine woodlands that are currently recognized by Forestry Commission in Scotland are found in very variable climates from the maritime west

coast to the more continental east (Mason, Hampson et al. 2004). Average temperature and precipitation conditions among the sites vary tremendously (Salmela, Cavers et al. 2010), but how such sharp environmental gradients and severe decrease in abundance of Scots pine have shaped the patterns of adaptive genetic variation is largely unknown. Marker studies demonstrated high levels of within and low levels of among-population variation (Forrest 1980; Kinloch, Westfall et al. 1986; Provan, Soranzo et al. 1998; Wachowiak, Salmela et al. 2011), but only a few studies on adaptive trait variation have been carried out and these were based only on a limited number of populations (Perks and McKay 1997; Perks and Ennos 1999). As a result, the current seed transfer guidelines of the species are based on the earlier marker studies (Forrest 1980; Kinloch, Westfall et al. 1986), and if local adaptation has occurred, the current guidance might allow seed transfers between two environmentally distant locations, thus increasing the risk of planting poorly adapted seedlings. In addition to extensive spatial heterogeneity, temporal variation for instance in the length of the growing season (Perry and Hollis 2005) and winter harshness (Harrison 1997) has been documented in the UK, and it is possible that such patterns are influenced, e.g., by North Atlantic Oscillation (NAO) which measures the strength of westerly winds across northern Atlantic (Stenseth, Mysterud et al. 2002). For example, sheep mortality on the island of St. Kilda in Scotland has been found to be higher in wet and warm winters which often occur at times when winter NAO indices are high (Milner, Elston et al. 1999). Such fluctuations in the environment could contribute to the maintenance of quantitative trait variation in species that have overlapping generations and groups unaffected by changes in the environment (Ellner and Hairston 1994). These criteria might be fulfilled in trees in which climate-related mortality has been found to be highest in young seedlings (e.g. Persson and Ståhl 1990). If the extent of temporal climate fluctuations varies among different parts of Scotland, more variable environments might support the maintenance of higher levels of quantitative trait variation in a long-lived species like Scots pine.

The aim of this study was 1) to examine how spatial and temporal climate conditions vary among 21 native pinewood sites from different parts of Scotland, 2) to test

whether population means of timing of bud flush observed in two common-garden glasshouse trials were associated with average temperature features at the origins of the populations, and 3) to explore whether temporal environmental heterogeneity could be a factor in determining the amount of within-population variation in this trait.

3.2 Materials and methods

3.2.1 Study populations

A total of 21 populations were sampled for this study, representing all parts of the species' range in Scotland (figure 3.1). Three populations represented each of the Forestry Commission native seed zones.
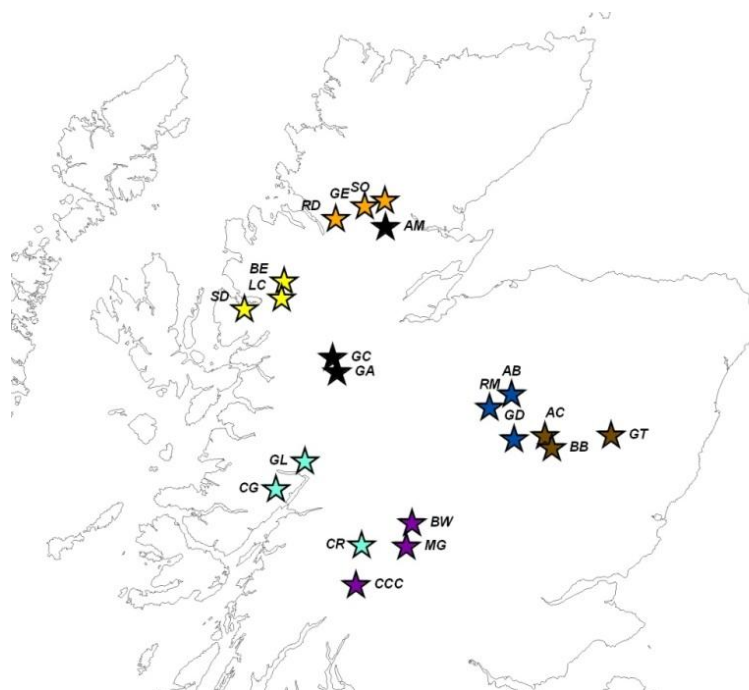
Figure 3.1 Map of the sampled populations, grouped according to their seed zones. Climatic features of the sites are shown in table 3.1.

Table 3.1 Populations included in the study, their coordinates (Lat., latitude; Long., longitude), mean altitude of the sampled sites within populations (Alt.), and average (1961-1990 or 1961-2000) climate features: growing season length (GSL; days), growing degree days (GDD: day degrees), February and July mean temperatures (FMT and JMT), and annual precipitation (AP).

Population	Lat.	Long. (W)	Alt. (m)	GSL	GDD	FMT (°C)	JMT (°C)	AP (mm)
Abernethy (AB)	57.21	3.61	343	211	990	1.1	12.7	1055
Allt Cul (AC)	57.04	3.35	475	145	513	-1.0	10.4	1018
Amat (AM)	57.87	4.60	145	214	892	1.2	12.3	1447
Ballochbuie (BB)	56.98	3.30	483	116	446	-1.7	9.5	1343
Beinn Eighe BE)	57.63	5.40	48	283	1329	3.7	14.2	2411
Black Wood of Rannoch (BW)	56.68	4.37	278	254	1138	2.1	13.5	1159
Coille Coire Chuilc (CCC)	56.42	4.71	271	226	928	1.6	12.3	2904
Conaglen (CG)	56.79	5.33	145	246	887	2.2	11.7	2592
Crannach (CR)	56.58	4.68	291	231	1019	1.8	12.6	2460
Glen Affric (GA)	57.26	4.92	268	210	769	0.9	11.6	1685
Glen Cannich (GC)	57.35	4.95	323	212	778	1.0	11.7	1983
Glen Derry (GD)	57.03	3.58	461	168	593	-0.5	11.3	1056
Glen Einig (GE)	57.96	4.76	67	242	1089	2.2	13.2	1463
Glen Loy (GL)	56.91	5.13	170	191	541	0.5	9.8	2156
Glen Tanar (GT)	57.02	2.86	334	235	1105	2.2	13.6	785
Loch Clair (LC)	57.56	5.36	124	277	1253	3.4	13.7	2790
Meggernie (MG)	56.58	4.35	306	223	916	1.1	12.0	1497
Rhidorroch (RD)	57.89	4.98	180	221	840	1.5	11.6	1778
Rothiemurchus (RM)	57.15	3.77	314	224	1087	1.4	13.1	1042
Shieldaig (SD)	57.50	5.63	77	273	1093	3.2	12.8	2385
Strath Oykel (SO)	57.98	4.61	99	257	1276	2.7	14.0	1215

3.2.2 Climate data

Long-term averages

UK Met Office long-term average (1961-2000) climate data (Perry and Hollis 2005) were used to make climatic profiles of the areas where the populations occur (table 3.1). Data for annual precipitation covered the period 1961-1990. Data were extracted for the length of the growing season (GSL), number of growing degree

days (GDD), mean February and July temperatures (FMT and JMT), and annual precipitation (AP). Climate data are available in 5×5 km grids and based on observations from weather stations and interpolation. Because no finer-scale climate data are available, variation in altitude among the sampled sites within populations was used as a proxy for within-population spatial heterogeneity and was expressed as a coefficient of variation (CV; (σ/mean)$\times$100).

Temporal variation

To investigate patterns of temporal variability in temperature variables among the origins of the populations, annual estimates of GSL, GDD, February and July temperatures (FT and JT) for the 5×5 km grids in the period between 1961 and 2000 were used. Using these 40-year data, mean absolute deviations (MAD; average of the deviations of the data points from their mean) of FT and JT, and *CV*s of GSL and GDD were calculated separately for each population. A combined estimate of temperature variability was calculated as the average of the MADs of February and July temperatures. North Atlantic Oscillation (NAO) index data was provided by the Climate Analysis Section, NCAR, Boulder, USA, at NAO Index http://www.cgd.ucar.edu/cas/jhurrell/indices.html.

3.2.3 Common-garden trials

Open-pollinated seed from 21 populations were sampled in March 2007 and used to establish two glasshouse based common-garden trials located in Edinburgh and Aberdeen. The two trials were set up by independent investigators and they therefore had rather different germination conditions and layout designs. In the Edinburgh trial located at CEH, seeds were sampled from four mother trees per population (i.e., 84 families in total) and sown on trays (75:25 compost type John Innes 1: sand) in June 2007 under common-garden glasshouse conditions. After germination, seedlings

were transferred to pots of size 0.62 l (diameter 11 cm, depth 9.6 cm) and kept under natural light conditions (glasshouse was shaded to avoid excess light) with watering applied 2-3 times per week during the growing season. No heating was applied during winter. Each family consisted of 40 progeny (~3,360 seedlings in total). The trial was divided into 40 blocks, each having one member from each of 84 families, and the order of the families within blocks was randomized. Following bud flush in 2008, the seedlings were transferred to larger pots (11 × 11 × 12 cm) and the compost was changed to John Innes 3. During the winter of 2008/2009, heating was used to protect seedlings from frost.

The Aberdeen trial was located at the Macaulay Institute (data provided by Glenn R. Iason). Cones were placed in a warm room (30°C) for 2 weeks so that they opened and seed could be extracted for germination. Seed from the individual trees were kept separate and were soaked in water for 3 h, then laid between sheets of damp paper towel placed in a cool room (3°C) for several weeks to break dormancy. Seeds were taken out of the cool room and left (wrapped in damp paper) in the laboratory at room temperature until they germinated. Germination took approximately 7 days and seeds from all the sampled families germinated at this time. On germination they were transplanted into potting medium in the glasshouse into 8 × 8 × 9 cm (0.4 l) pots (LBS horticulture, Colne, Lancashire UK). Each population consisted of ten families with eight progeny/family, and the trial was divided into 40 trays with 42 plants per tray, each tray containing two plants from a different mother from each population. Watering was applied automatically and no artificial light was used.

In the Edinburgh trial in 2008, timing of bud flush was scored twice weekly between March 23 and May 9. In the Aberdeen trial, scoring was done once a week, starting on March 31 and ending on May 27. In both trials, bud flush was considered to have taken place when new green needle tips started to emerge from the apical bud, and this was expressed as the number of days since the first scoring date. Bud flush was scored similarly in the Edinburgh trial in 2009. The first bud flush was observed on April 2 and the last one on June 1.

3.2.4 Statistical analyses

Climate variation

To examine spatial variation in average and temporal climate variables, long-term averages (GSL, GDD, FMT, JMT, AP) and estimates of temporal variability (MADs for FT and JT, *CV*s for GSL and GDD) were regressed against the longitude, latitude, and altitude at each population's origin. Linear regression was also used to test for an association between annual mean FTs and February NAO indices. Correlation analysis was used to examine relationships between the populations' coordinates and their altitude, and between different climate variables.

Quantitative trait data

Testing for group differences

Data from the Edinburgh trial were analysed using nested analysis of variance (ANOVA), with populations considered fixed and families within them and blocks random factors. Unbalanced ANOVA was applied to the data from the Aberdeen trial. Variance components due to populations, families, and blocks were estimated using a mixed model (REML) approach. Correlation analysis was used to test whether similar trends were observed in the two trials.

Associations between population means and climate averages at home site

To investigate associations between the locations of the populations, their climate, and variation in timing of bud flush, population means in the two trials were regressed against their longitude, latitude, altitude, and average temperature estimates (GSL, GDD, FMT, JMT) at origin.

Amount of variation in timing of bud flush

Due to common-garden environments with randomized block designs, it was assumed that the variation observed in each trial was due to genetic causes and that possible differences among populations and families were due to varying levels of genetic variation. To measure the amount of variation in the trait within populations and families, *CV*s were calculated for each group ((σ/group mean)$\times$100) separately in the two trials. In order to estimate the amount of among-family variation, REML was used to calculate variance components due to families within each population and trial. Among-family *CV*s of timing of bud flush were then calculated as ($\sqrt{}$(variance component due to families within populations)/population mean)$\times$100. Correlation analysis was used to test whether similar patterns of variation among population *CV*s were observed in the two trials.

Association between trait variability within populations and altitude

To examine how the site of the experiment (Edinburgh or Aberdeen) affected population *CV*s, analysis of covariance (ANCOVA) was carried out, with *CV*s as the variate, site as the main factor, and altitude as a covariate. In addition, linear regression analyses with population and family *CV*s and altitude were carried out separately within each trial. To examine whether differences in the range of altitudes sampled within each population contributed to the amount of variation in the trait,

population *CV*s were regressed against the estimate of spatial heterogeneity within each population. The possibility of among-family variation being influenced by altitude was tested by regressing among-family *CV*s of each population against altitude.

All analyses were carried out using GenStat Ver. 13.1.0.4470.

3.3 Results

3.3.1 Spatial climate variation

The sampled populations were located across a latitudinal and longitudinal gradient 1.56° and 2.77°, respectively. The altitude of the populations decreased strongly towards the west (r=-0.8050, p<0.001). Among the populations, average GSL ranged from 116 days in BB to 283 days in BE, and average GDD from 446 to 1,329 dd in the same populations (table 3.1). Average annual rainfall ranged from 785 mm in GT to 2,904 mm in CCC. GSL decreased towards higher altitudes (β_0=283.6, β_1= -0.2493, p<0.001, R^2=62.8%), and a similar but weaker association was observed with GDD (β_0=1231.5, β_1=-1.227, p=0.001, R^2=39%). Higher altitude sites were also associated with lower FMT (β_0=3.541, β_1=-0.00839, R^2=65.9%) and JMT (β_0=13.570, β_1=-0.00521, p=0.013, R^2=24.6%). Annual precipitation was best associated with longitude and showed a decreasing trend from west to east (β_0= -1,275, β_1=668, p<0.001, R^2=62%).

3.3.2 Temporal climate variation

Climate differed markedly from year to year. For example, annual mean GSL and GDD of the sites occupied by the 21 pinewoods showed extensive temporal

fluctuation in the period 1961-2000 (figure 3.2a). Mean GSL varied between 174 days in 1968 and 271 days in 1989, while the lowest mean GDD (756 dd) was reached in 1974 and the highest (1,167 dd) in 1995. Temporal variability was also found in monthly winter and summer temperatures. The range of JTs was 9.9 °C in 1965 and 14.9 °C in 1983, and annual means were significantly correlated with GSL (r=0.4633, p=0.0026) and GDD (r=0.6409, p<0.001) in the same year. Mean FTs varied between -2.4 °C in 1963 and 5.9 °C in 1998, and were found to be influenced by the North Atlantic Oscillation (NAO), with colder temperatures being associated with lower NAO indices (figure 3.2b; β_0=1.2863, β_1=0.5789, p<0.0001, R^2=32.6%).

Populations from different parts of Scotland were found to experience different levels of temporal variation in these climate features. Mean MAD of FT and JT increased with ascending altitudes (figure 3.2c; β_0= 1.0315, β_1=0.0007176, R^2=77%), while in GSL and GDD, temporal variability increased very little from altitudes of 48 to 343 m, but was higher at the three sites located above 450 m (figure 3.2d).

3.3.3 Timing of bud flush

In the Edinburgh trial in 2008, population means in timing of bud flush varied between 11 days in GA, AC, GD, BB, and GT, and 18 days in SD and RD. ANOVA indicated significant differences among populations (p=0.058), families within populations, and among blocks (table 3.2a). The variance component due to differences among families within populations (15.40) was approximately five times larger than that of populations (2.98). In 2009, population means varied between 22 days in SD and 31 days in AM, and significant differences were found between populations, families within populations and blocks (table 3.2b). The variance component due to families in 2009 (10.86) was approximately four times larger than that of populations (2.5). In the Aberdeen trial, the range of population means was from 16 days in AC and GL to 21 days in CG, BE, SO, MG, SD, LC, and AB. Significant differences were observed among populations, families within

populations, and blocks (table 3.2c). The variance component due to families (5.88) was approximately four times larger than that of populations (1.45).

Population means between the two trials in 2008 were significantly correlated (r=0.5068, p=0.0190).
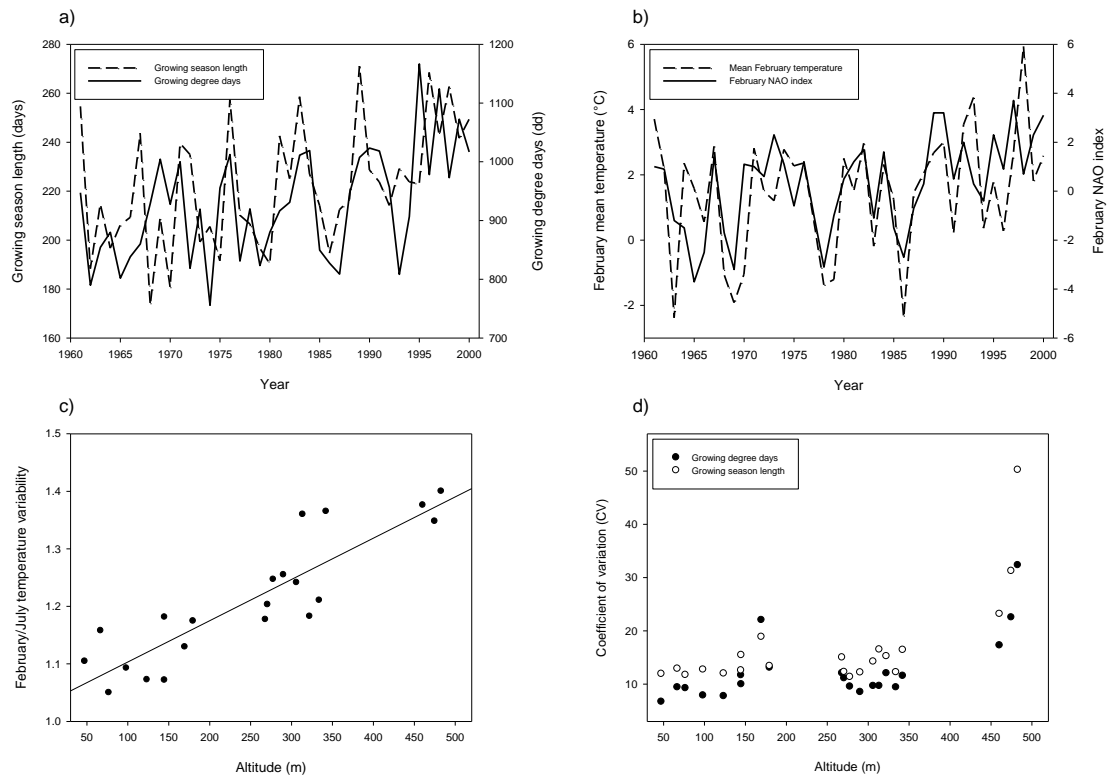
Figure 3.2 a) Temporal variation in mean annual GSL and GDD; b) temporal variation in annual FTs and February NAO indices; c) relationship between the altitudes of the 21 native pinewood sites and variability of winter and summer temperatures, expressed as the average of MADs of FT and JT; d) *CV*s of temporal variation in GSL and GDD plotted against site altitude. The climate data used cover the period 1960-2000. In a) and b), annual means were calculated over the 5×5 km grids within which the 21 pinewood sites are located.

Table 3.2 ANOVA results for timing of bud flush in the Edinburgh in a) 2008 and b) 2009, c) in the Aberdeen trial in 2008, and d) ANCOVA for the 2008 data.

a) Edinburgh trial: 2008

Source of variation	df	MS	*F-ratio*	*p*-value	Variance component
Population	20	1153.35	1.70	0.06	2.98
Families w ithin populations	63	679.52	13.50	<0.001	15.39
Block	39	176.66	3.51	<0.001	1.50
Residual	3120	50.35			50.40

b) Edinburgh trial: 2009

Source of variation	df	MS	*F*-ratio	*p*-value	Variance component
Population	20	921.04	1.76	0.04	2.50
Families w ithin populations	63	523.69	5.95	<0.001	10.86
Block	39	610.07	6.93	<0.001	6.11
Residual	3051	88			87.99

c) Aberdeen trial: 2008

Source of variation	df	MS	*F*-ratio	*p*-value	Variance component
Population	20	183.26	2.18	<0.01	1.45
Families w ithin populations	188	83.96	2.01	<0.001	5.88
Block	39	221.37	5.29	<0.001	5.09
Residual	1216	41.87			42.26

d) ANCOVA

Source of variation	df	MS	*F*-ratio	*p*-value
Site	1	4315.4	83.54	<0.001
Covariate (altitude)	1	1835.39	35.53	<0.001
Residual	39	51.66		
Total	41			

Covariate coefficient (s.e.): 0.0511 (0.00875)

Spatial patterns of variation in timing of bud flush

In the Edinburgh trial, population means of timing of bud flush in 2008 were significantly associated with altitude at their origin. Low-altitude populations generally flushed later than those from higher locations, and altitude explained 24.2% of the variation among population means (β_0=16.75, β_1=-0.01054, p<0.014). Altitude of the populations was negatively correlated with average GSL (r=-0.80, p<0.001) and GDD (r=-0.65, p=0.0015), and higher R^2's are obtained when using these climate variables instead of altitude. Earlier bud flush occurred in populations from areas with shorter GSL (β_0=5.67, β_1=0.0383, p=0.004, R^2=33.3%), fewer GDD (β_0=8.17, β_1=0.00646, p=0.003, R^2=35.6%), lower FMT (β_0=12.502, β_1=1.133, p=0.005, R^2=31.4%), and JMT (β_0=0.65, β_1=1.100, p=0.010, R^2=26.4%). Population means in 2009 were not significantly associated with any of these factors.

A similar trend with altitude was also found in the Aberdeen trial (β_0=20.149, β_1= -0.00467), but the association was not statistically significant at the 0.05 level (p=0.085, R^2=10.3%). Significant associations were obtained when temperature estimates were used instead: sites with shorter GSL (β_0=13.47, β_1=0.02612, p<0.001, R^2=41.6%), fewer GDD (β_0=15.24, β_1=0.00433, p<0.001, R^2=42.7%), lower FMT (β_0=18.131, β_1=0.773, p=0.001, R^2=39.2%), and JMT had earlier bud flush (β_0=9.18, β_1=0.821, p=0.001, R^2=40.8%).

Within-population variability

In the Edinburgh trial in 2008, population *CV*s varied between 44% in RD, BE, and SO, and 79% in BB. In the Aberdeen trial, *CV*s varied from 21% in CG to 56% in AC and GD. ANCOVA revealed significantly larger *CV*s in the Edinburgh trial (mean 57.236%) than in Aberdeen (mean 36.936%; table 3.2d), but the population *CV*s were significantly correlated between the two trials (r=0.5139, p=0.0172). Altitude also accounted for a significant proportion of the variation observed among *CV*s, and the positive combined regression coefficient (β=0.0511) indicated that

larger CVs were observed at higher altitudes (figure 3.3). The combined coefficient was close to those observed when regression analyses were carried out separately within each trial (for the Edinburgh trial: $\beta_0=44.33$, $\beta_1=0.0524$, $p<0.001$, $R^2=44\%$; Aberdeen trial: $\beta_0=24.69$, $\beta_1=0.0498$, $p<0.001$, $R^2=46.1\%$). Both regressions were strongly influenced by the three highly variable high-altitude sites. When excluding them, linear regressions in both trials became statistically non-significant at the 0.05 level ($p=0.104$ in Edinburgh, $p=0.084$ in Aberdeen), although the trends remained positive in both cases and R^2's above 10% ($R^2=10.4\%$ in Edinburgh, $R^2=12.3\%$ in Aberdeen).

Further regression analyses were carried out to examine possible causes of differences among population CVs. Increasing spatial heterogeneity within populations did not account for larger CVs at high altitudes (the Edinburgh trial: $\beta_0=64.29$, $\beta_1=-0.319$, $R^2=25.7\%$, $p<0.011$; the Aberdeen trial: $\beta_0=42.46$, $\beta_1=-0.290$, $R^2=18.1\%$, $p<0.031$). However, among-family CVs in the Edinburgh trial were positively associated with altitude, and the regression explained 27% of their variation ($\beta_0=11.25$, $\beta_1=0.0596$, $p=0.009$); the trend in the Aberdeen trial was similar in direction ($\beta_0=2.54$, $\beta_1=0.0256$, $R^2=10.4\%$), but non-significant at the 0.05 level ($p=0.085$). When within-population variability in the Aberdeen trial was calculated as the CV among the ten family means, a significant correlation between REML estimates and among-mean CVs was observed ($r=0.8457$, $p<0.001$) and a linear regression with altitude was significant ($\beta_0=9.52$, $\beta_1=0.03333$), explaining 34.7% of the variation ($p=0.003$).

In 2009, population CVs in the Edinburgh trial ranged from 32% in MG to 45% in BB and LC, and no significant associations between them and altitude were found.
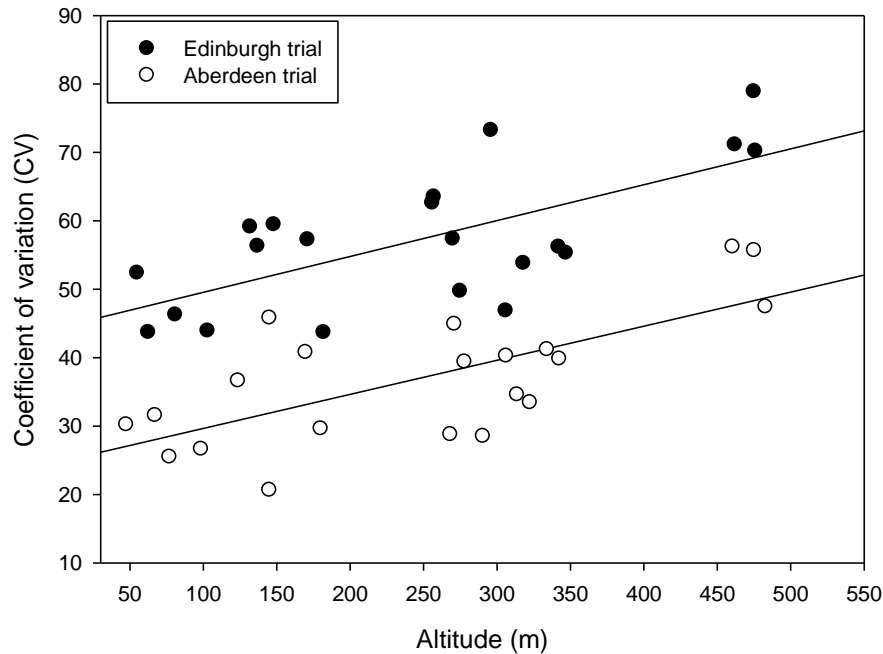
Figure 3.3 Relationship between site altitude and *CV*s in timing of bud flush in 2008 among 21 populations in the two trials. In the Edinburgh trial: β_0=44.33, β_1=0.0524, p<0.001, R^2=44%; in the Aberdeen trial: β_0=24.69, β_1=0.0498, p<0.001, R^2=46.1%.

Within-family variability

The amount of variation within families was also found to be associated with altitude. In the 2008 data from the Edinburgh trial, the least variable family was from SO (*CV*=25%), and the most variable family was from AC (*CV*=113%). A positive association between altitude at the families' home sites and family *CV*s was discovered, and a linear regression (β_0=41.80, β_1=0.0488) explained 16.6% of the variation among *CV*s (p<0.001); without the three highest-altitude sites (12 families), R^2 fell to 3.9% but the association remained marginally significant (p=0.053). In the Aberdeen trial in 2008, family *CV*s varied between 0% in ten families from seven populations and 105% in a family from AC. Linear regression (β_0=23.84, β_1=0.03544) explained 6.5% of the variation among within-family variability (p<0.001), but was non-significant (p=0.483) when the three highest-altitude sites

were excluded. In 2009, family *CV*s varied between 21% in AB1837 and 57% in CR1888, but their association with altitude was not significant.

3.4 Discussion

This study examined how timing of bud flush varies among native Scots pine populations from different parts of Scotland using data from two common-garden trials. Climate data suggest that spatial climate heterogeneity among the studied populations is high, with clear gradients between different temperature features of the sites. Bud flush at the beginning of the second growing season was generally found to occur earlier in populations from high-altitude areas which had shorter GSL and fewer GDD, but most of the variation was found within populations. Differences were also found in the amount of variation in this trait, and *CV*s on population and family levels showed an increasing trend with ascending altitude of sites. Analyses of temporal climate variation suggest that these sites also experience more fluctuating temperature conditions which could contribute to the maintenance of variation in this quantitative trait. However, no such trends were found in the data collected in 2009.

3.4.1 Spatial and temporal climate variability

Although the geographic area of native pinewoods in Scotland is small, spatial environmental variation across the Scottish sites is extensive (Mason, Hampson et al. 2004). The general features of climate variation among the 84 native pinewood sites have been described previously (Salmela, Cavers et al. 2010), and the populations included in this study provide a good representation of the range of temperatures and rainfalls experienced by the sites. For instance, BB located in the eastern Highlands and at a high altitude (~483 m) has a short average GSL (116 days), the coldest FMT (-1.7°C) and the lowest JMT (9.5°C), while BE in the west, located at an altitude of ~48 m, has corresponding values of 283 days, 3.7 °C, and 14.2 °C. These differences

probably reflect variation in both distance from the Atlantic Ocean and in topography. Based on the patterns of spatial climate variation, Salmela, Cavers et al. (2010) concluded that the current seed zones of Scots pine do not fully represent environmental variation among populations.

Analyses of among-year variation in climate suggest that the environments are not temporally stable, and clear fluctuations were discovered in annual summer and winter temperature features. Such patterns are common features of environmental variation in natural ecosystems (Vasseur and Yodzis 2004) and they can have various effects on population dynamics depending on the type of the fluctuation and species (e.g. Ruokolainen, Lindén et al. 2009). An association between FTs and February NAO indices in the period 1961-2000 was also observed. The NAO describes the pressure difference between the atmospheric low pressure over Iceland and the subtropical high-pressure over Azores, and is known to have a strong influence especially on winter temperatures in the North Atlantic (Ottersen, Planque et al. 2001). High indices are associated with increased precipitation and higher temperatures, while cold winters often occur at the time of very negative winter NAO indices. Variation in the NAO has been linked to various biological processes from fluctuations in population size to timing of breeding in animals (reviewed in Stenseth, Mysterud et al. 2002), and in plants, earlier flowering has often followed warm and wet winters (Post and Stenseth 1999). Winter NAO indices have been traced back to 1864 (Hurrell 1995), and assuming that they can be used as proxies for winter temperatures, it is likely that similar fluctuations to those observed between 1960 and 2000 have also taken place earlier.

Interestingly, although all populations in Scotland appear to be influenced by these fluctuations, the extent of this temporal variability in climate shows spatial variation. In our set of populations, most temporal variation in summer and winter temperatures is observed at the highest-altitude sites located in eastern Highlands. The observed patterns are unlikely to be caused by altitude alone, but also by for instance continentality which is influenced by distance from sea. Associations between temporal variability and altitude are probably caused by the fact that in our sample of

populations, longitude is strongly negatively correlated with site altitude, meaning that low-altitude sites are found in more oceanic climates close to the west coast where within and among-year climate variation is smaller than in more eastern and continental high-altitude pinewood sites. Previously, similar observations have been made by Vasseur and Yodzis (2004) who found in their analysis of 152 time series of various environmental variables from different parts of the world that coastal areas can be buffered against high-frequency changes that are typical of inland sites. However, variation in GSL and GDD in Scotland in the period 1961-2000 suggests that there are only minor differences in temporal variation among sites at altitudes below ~350 m.

The climate data used here have limitations which should be borne in mind when interpreting the results. The data do not represent actual measurements for the individual pinewood sites, but are values estimated for the centres of 5×5 km grids within which the sites are located, and are based on a temporally and spatially varying number of weather stations and interpolation, the precision of which varies depending on the variable being estimated (Perry and Hollis 2005). In addition, the sampled sites are located at various distances from grid centres, and therefore, the actual conditions experienced by pinewoods might differ from the estimated grid value due to for instance topographic variation within grids. Thus, the climate data should only be considered as approximate proxies for the site conditions, and further interpolation would be required to allow finer-scale estimates. However, indirect evidence of temporal variability in summer temperatures has been found in patterns of variation in latewood density chronologies among five Scottish pinewoods (Hughes, Schweingruber et al. 1984). Wood density among years fluctuated in a similar way in all populations, but the extent of temporal variation was largest in BB, and lower at sites located closer to the west coast.

3.4.2 Variation in timing of bud flush

Evidence of adaptive genetic differentiation among populations from diverse climates was found in 2008 when bud flush generally took place earlier in populations from high-altitude sites which experienced shorter growing seasons and colder winters. This could be an adaptation to a shorter growing period, enabling a quick response to rising spring temperatures. Similar trends were observed in the two trials although sampling and scoring intervals were somewhat different. Also, bud flush started in both glasshouses around the same time (late March). However, a larger proportion of variation was due to differences among families within populations, indicating that the trait is genetically controlled and that within-population and thus evolutionary potential is maintained. Phenological differentiation among Scots pine populations has been extensively studied in continental European populations, and it has been shown that growth cessation in common-garden trials generally occurs earlier in trees from areas with shorter growing seasons and earlier onset of winter (Oleksyn, Tjoelker et al. 1992; Hurme, Repo et al. 1997). Less attention has been paid to growth initiation in spring. In this study, significant associations of population means with environmental variables suggest that genetic differentiation in response to environmental differences has occurred among native pinewoods, and that such patterns should be taken into account in the seed transfer guidelines in order to minimise the risk of planting seedlings too far from their home environments. However, for a more comprehensive understanding of adaptation, variation in other environment-driven traits (e.g. cold hardiness and its timing, cessation of growth) and correlations among them should also be assessed.

Bud flush occurs in spring after genetically determined chilling and heat sum requirements have been met (Dougherty, Whitehead et al. 1994; Aitken and Hannerz 2001), and the observed differences among populations and families most likely reflect variation in the required heat sum rather than in the required number of days. Environmental cues for growth initiation can vary among populations (e.g. Dougherty, Whitehead et al. 1994), and in Scots pine, a Scottish population was found to have a longer chilling and a higher heat sum requirement compared to

Northern European populations, indicating a mechanism preventing initiation of growth under mild winter conditions (Leinonen 1996). It is likely that chilling requirements of the populations were fulfilled during winter before the onset of the second growing season as there was no external temperature treatment being applied to the trial. The population-specific chilling and heat sum requirements cannot be estimated based on these results, but they could be expected to vary due to extensive temperature variation within Scotland. Considering that our results suggest only small differences among groups when grown in a glasshouse, more frequent scoring and placing seedlings outdoors (to decrease the rate of heat sum accumulation) or controlling the temperature indoors might reveal bigger population differences.

Previously, Perks and Ennos (1999) observed that in a common-garden trial of seven-year old seedlings from four native pinewoods, AB had a significantly later bud flush than the three other populations, two of which (BW, GA) were also included in this study. However, this pattern could not be linked to any environmental factor due to a limited number of sampled populations. Variation in timing of bud flush or height growth initiation has been studied in many early provenance trials of Scots pine: in early studies Langlet (1936) had already noted that growth initiation in Scandinavian seedlings was earlier than in those from Central Europe, and similar observations were also made by Dietrichson (1961). Later studies reported variation across much wider geographic scales spanning hundreds or even thousands of kilometres. In a study of eight-year old trees from 36 mainly European provenances growing in Nebraska, height growth in spring was reported to have started earlier in northern European populations than in those from more central and southern parts of the continent (Read 1971). In an extensive trial consisting of 14-year old trees from 108 Eurasian populations growing in Michigan, bud flush started earliest in Siberian and northern Finnish populations, and was latest in those from southern and Central Europe (Steiner 1979). No major differences were observed among populations located south of Fennoscandia. Scotland was represented by three populations, and timing in those was later than in populations from the same latitudes in continental Europe. Studying trees aged ~60 years in two Scots pine series in southern Finland, Beuker (1994) found differences in the

required heat sum among four Finnish and one Russian population, the requirement being lowest in trees from North Finland (67°N) and highest in the Russian population located at 60°N. Observations were made during three years in both experiments, and the pattern of timing remained similar between years. However, the difference between the overall means of the earliest and latest-flushing populations was small (57 dd). It has been suggested that populations are often more differentiated in growth cessation than in its initiation in spring (Aitken and Hannerz 2001), and it would also be of interest to examine whether Scottish populations follow a similar trend and if there is a correlation between timing of growth initiation and cessation. It is possible that in species that are distributed across spatially heterogeneous environments the driving force of differentiation in growth initiation varies among regions. In North American whitebark pine, timing of bud flush was associated with the length of the frost-free period only in the northern part of its range, while in other locations it appeared to be more linked to e.g. drought avoidance (Bower and Aitken 2008). The wide Eurasian range of Scots pine spans hugely variable environments from the Atlantic Coast to Central Siberia and Asia along which both temperature and precipitation variation is extensive, and it is possible that the same trait is influenced by different environmental factors in various parts of the range in Scots pine.

3.4.3 Within-population variation in timing of bud flush

In addition to variation among population means, different levels of variation in timing of bud flush within populations were found in the 2008 data. Although the number of families within populations and progeny within families were different in the two trials, in both cases the three highest-altitude sites – AC, BB, GD – exhibited large population CVs, with CVs showing a positive correlation with altitude of sites. Interestingly, climate data suggest that temporal climate variation varies along the same gradient in this set of populations, with highest levels of fluctuation occurring at the three highest-altitude areas.

While variation in trait means among populations from diverse environments has been assessed on numerous occasions (reviewed in Howe, Aitken et al. 2003; Savolainen, Pyhäjärvi et al. 2007; Aitken, Yeaman et al. 2008), few studies have examined how the level of variation in quantitative traits within populations varies. Notivol, García-Gil et al. (2007) discussed variation in additive genetic coefficients of variation (CV_A) in growth rhythm traits among two Finnish and Spanish Scots pine populations in the context of different selection intensities in different populations. In some traits (e.g. timing of bud set), the northern Finnish population, located close to the range edge, showed higher CV_A compared to the southern Finnish population, but in other traits (e.g. duration of growth) the trend was reversed. The factors causing these patterns remain unknown, but differences among traits are not surprising because their environmental driving forces of adaptation might differ. Our results suggest that among-family variation could be increased at high-altitude sites, thus contributing to the overall increase in quantitative genetic diversity. Moreover, the most variable families were from the highest-altitude sites.

One possible explanation for the observed patterns is the expression of "cryptic genetic variation" in a novel environment (e.g. Hoffmann and Merilä 1999). Some of this variation might be hidden (neutral) in the native environments of the populations where different genotypes are exposed to similar environments and thus similar selection pressures. In a different environment such hidden variation could be expressed. For example, Conner, Franks et al. (2003) found that in wild radish (*Raphanus raphanistrum* L.), estimates of additive genetic variance for floral traits were different under glasshouse and field site conditions, and generally higher additive and smaller environmental variances were observed in the glasshouse experiment. It is possible that population differences found in this study are due to the environment being more novel to some populations (e.g. those from high-altitude sites), thus resulting in different levels of hidden variation being expressed. However, all populations were exposed to an artificial environment, and determining whether this treatment affected populations in different ways is difficult. Smaller *CV*s were found in the Aberdeen trial which could be due to a different growing environment. However, the association between altitude and population *CV*s was

similar to that observed in the Edinburgh trial, and it is possible that the differences between the two trials were also due to different sampling and scoring intervals (twice weekly in Edinburgh, once weekly in Aberdeen).

Other explanations for the patterns can also be examined. One premise on which the foregoing discussion is based is that it is assumed that the patterns of quantitative trait variation observed under glasshouse conditions still reflect variation in the levels of additive genetic variance (V_A) in this trait among populations. Although maternal effects have been shown to have some short-lived influence on some adaptive traits in Scots pine, environmental conditions during seed development have been found to have no effect on timing of bud set after the first growing season (Dormling and Johnsen 1992; Ruotsalainen, Nikkanen et al. 1995). Progeny from controlled crosses of northern Finnish parent trees growing under southern Finnish conditions have been found to perform as well in the north as local progeny (Rousi 1983), and crosses between known parents also show evidence of additive inheritance for bud set and cold hardiness (Hurme, Repo et al. 1997), suggesting that these adaptively significant traits exhibit Mendelian inheritance. Similar analyses have not been carried out for timing of bud flush in Scots pine, but in other tree species, the trait has been found to be under strong genetic control (Howe, Aitken et al. 2003). A larger number of families within populations would be required for better estimates of variation due to additive effects.

Assuming that these results reflect variation in the level of adaptive genetic diversity within populations, the following explanations for the patterns can be considered:

1. **In general, high-altitude sites are genetically more diverse, and other populations have lost diversity as a result of e.g. colonization and heavy exploitation.** Molecular marker studies do not support this explanation as they do not show any major differences in diversity across Scotland or compared to more continuous continental pinewoods, either in allozymes (Kinloch, Westfall et al. 1986), chloroplast DNA (Provan, Soranzo et al. 1998), or candidate gene variation (Wachowiak, Salmela et al. 2011), and

among-population differences account only for a small percentage of total genetic variation. Pinewood size (according to Mason, Hampson et al. 2004) is not associated with amount of quantitative variation ($p>0.7$ in both trials), and data from the mating system analysis (Chapter 2) suggest high and similar levels of outcrossing in these populations.

2. **Increased genetic diversity at high altitudes is due to an admixture of genetically diverged lines.** Admixture should also result in increased diversity at molecular markers but these show very similar levels of variation among populations. It is likely that native Scottish pinewoods are of at least two different origins, but so far, only some western populations have been found to harbour unique maternally-inherited mitochondrial DNA (mtDNA) variation (Sinclair, Morman et al. 1998). This does not exclude the possibility of ancient contact zones further east as the number of studied polymorphic sites is currently very limited, but it is not clear for how long the increased diversity resulting from admixture would be expected to persist in a mostly outcrossing conifer. More variable mtDNA polymorphisms should be assessed to study postglacial colonization routes in more detail.

3. **Gene flow from other populations is more frequent at high altitudes.** Long-distance pollen-mediated gene flow is significant in wind-pollinated trees (Smouse and Sork 2004) such as Scots pine, and gene flow among spatially heterogeneous sites has been suggested as a mechanism for maintenance of variation in another *Pinus* species (Yeaman and Jarvis 2006). However, estimates of average distance of pollen flow suggest that the great majority of the fertilizing pollen comes from local trees (Smouse and Sork 2004; Savolainen, Pyhäjärvi et al. 2007); for example, in an isolated Spanish mountain population of Scots pine, 4.3% of fertilizing pollen originated from at least 30 km away (Robledo-Arnuncio and Gil 2005), but it is not known how much temporal variation these patterns could have. Gene flow could be expected to contribute more to within-family than among-family variation in the populations because offspring resulting from matings between distant

parents might not be as well adapted to their home site as those with local parents (Burczyk, DiFazio et al. 2004). Female flowering starts earlier than male flowering in Scots pine (Sarvas 1962; Chung 1981), and for example in northern Finland, southerly winds bring pollen from the south at a time when local female strobili are receptive but male strobili are still immature (Varis, Pakkanen et al. 2009). Progeny from north × south crosses have been shown to have higher mortality in northern conditions than those from crosses between local parents (Pulkkinen, Haapanen et al. 1995). In Scotland, the coverage of Scots pine plantations is much wider than that of native pinewoods (Mason, Hampson et al. 2004), but their exact origin is often unknown and it is not known whether they contribute to gene flow via pollen (Salmela, Cavers et al. 2010). Very low among-population differentiation among pinewoods (Kinloch, Westfall et al. 1986; Provan, Soranzo et al. 1998) suggest that at least historically, gene flow across Scotland has been strong, but currently no data are available on the reproductive dynamics among and within the studied populations. Based on the patterns observed in Finland and on the climate variation among the populations in Scotland, it is likely that population reach their reproductive phases at different times, but whether this contributes to long-distance gene flow depends also on wind conditions at the time of pollen shedding.

4. **Spatial heterogeneity is higher at high-altitude sites, supporting the maintenance of variation within populations.** How climatic conditions vary within populations could not be determined in detail, but regression analysis was carried out to examine whether higher population *CV*s were associated with larger altitudinal variation among the sampled sites within populations. In both trials, population *CV*s showed a decreasing trend towards higher estimates of spatial heterogeneity within populations. These patterns do not support this hypothesis, but it is possible that altitudinal variation does not reflect within-population variation in specific climate variables. A closer examination of potential variability in spatial heterogeneity would require further interpolation of the climate data to finer geographic scales.

5. **Temporal climate variation contributes to the maintenance of higher levels of variation.** This hypothesis is supported by the patterns of temporal climatic variation, but based on these data it is not possible to separate between the potential contributions of spatial and temporal environmental variation and/or their interaction. If the majority of fertilizing pollen is assumed to be of local origin, high within-family *CV*s at high altitudes could be due to local matings between more genetically diverse parents.

3.4.4 Temporally fluctuating environment and adaptive genetic diversity

How adaptive variation is maintained in natural populations remains a major question in evolutionary biology studies, and the topic has been investigated mainly from a theoretical point of view (Barton and Turelli 1989; Barton and Keightley 2002). Several factors are likely to contribute to genetic variation, and separating between the contributions of different factors is challenging. Spatially varying selection and gene flow are thought to be major factors in forest trees (Howe, Aitken et al. 2003), but the potential role of temporal fluctuations has not been explored in more detail. Natural environments are characterised by fluctuating conditions, the extent of which can vary depending for instance on the proximity of large bodies of water (Vasseur and Yodzis 2004), and in a recent review, Bell (2010) stated that similarly fluctuating selection is common and often strong in nature.

Quantitative genetic models suggest that temporally varying selection pressure could allow the maintenance of quantitative genetic variation in species that have overlapping generations and if selection acts only on a proportion of the individuals (Ellner and Hairston 1994). This might be the case in long-lived forest trees in which selection is very strong at the early stages of development and climate-related mortality in adult trees aged 20 years or more is often low (Persson and Ståhl 1990; Petit and Hampe 2006; Niinemets 2010). Winter harshness could be a strong

selective force, and climate data suggest that annual variation for instance in FT can be large and that at high altitudes that experience lowest summer and winter temperatures, fluctuations are more extreme. Single-year mortalities in northern Swedish Scots pine trials have been found to be increased by extreme winter conditions, although mortality does not necessarily occur immediately (Eiche and Andersson 1974). In Scotland, Cannell (1985) estimated that the frequency of potentially damaging spring frosts to Sitka spruce was highest in the uplands and lowest on coastal regions. Assuming that trees become more tolerant of stress as they age, it is possible that although progeny from different years might be exposed to the same environmental conditions, there are differences among them in mortality, depending on the age of the seedlings. If winter temperatures fluctuate randomly, the phenotypic range of young seedlings that survive might differ from year to year. Currently, there are no data on the environmental conditions during the establishment period of the sampled mother trees, but variation in winter NAO indices between 1864 and 2003 suggests that several periods have been characterised by persisting negative or positive NAO phases and cold or warm winters (Hurrell 1995; Stenseth, Mysterud et al. 2002) so that selection intensity might be occasionally relaxed or strengthened for periods essential for young seedling survival.

3.4.5 Effects of environmental fluctuations on reproduction

Another process besides selection that is strongly influenced by environmental conditions in Scots pine is reproduction. Temporally fluctuating climate is also characteristic of Lapland in North Finland where Scots pine forms the tree line (Pohtila 1980). Reproduction in Scots pine from the development of regenerative buds to seed maturation takes three years and each developmental phase has heat sum requirements (Hilli, Hokkanen et al. 2008 and references therein). For example, it has been suggested that about 850 day degrees are needed for 50% of the seeds to mature in Lapland (Sarvas 1970; Hilli, Hokkanen et al. 2008). Temporal and within-population variation in pollen production is also more variable in northern environments (Koski and Tallqvist 1978). Because of the unstable environment,

good seed years occur only rarely at the tree line, resulting in infrequent recruitment events. This could also be the case at high altitude and low-temperature sites in the Scottish Highlands. The genetic structure – both adaptive and neutral – of progeny could vary temporally if a fluctuating climate results, e.g., in more within-population variation in reproductive success. Fluctuations in cone production in eastern Scotland have been reported before (McNeill 1954), but extensive comparisons between various parts of Scotland have not been carried out. Analysing age structure of old trees in native stands could give some indication of past recruitment events: in Sweden, such events have been suggested to occur less frequently in northern pinewoods (Ågren and Zackrisson 1990).

3.4.6 Effects of temporal fluctuations on genetic structures

Infrequent recruitment events and varying selection intensity could potentially be reflected in cryptic population substructures, with allele frequency differences among trees established at different times. Genetic variation is often thought to be randomly distributed within tree populations, but recently the importance of examining such structures among populations from environmentally diverse areas has been recognized especially when searching for associations between marker polymorphisms and phenotypic variation (Jansson and Ingvarsson 2010). Peripheral and disjunct Sitka spruce populations have been found to be more structured than central populations (Gapare and Aitken 2005), and while differences in tree density might account for a significant proportion of this variation, climate could also influence these patterns if more peripheral populations occur in more fluctuating environments. Also, in a study of three populations of Scots pine in Sweden, with two or three stands within each population, small but significant differences at allozymes among stands were observed only in the population located at high altitudes above 675 m close to the Scandinavian Mountains (Gullberg, Yazdani et al. 1985). Similar temporal fluctuations in climate and recruitment could partly explain such a pattern, but additional intensive sampling among and within populations would be required for a more in-depth look at possible structuring.

In Scots pine in Scotland, differences in the decay of linkage disequilibrium (LD) among three geographical groups (eastern, western, and southern) have been reported (Wachowiak, Salmela et al. 2011), and the eastern group, which included the high-altitude pinewoods of this study, was found to have more extensive LD compared to the southern and the western groups. In trees, such patterns have been discussed mostly in the context of demographic processes such as ancient bottlenecks, population expansions, and admixture of diverged lines (Lascoux, Pyhäjärvi et al. 2008), but increased LD could also be due to population substructures if different within-population cohorts that vary in allele frequencies are sampled as one group (Nei and Li 1973). The lower LD in the more maritime western group might be due to more stable environmental conditions that are closer to an equilibrium state. More intensive within-population sampling and a larger number of markers would be required for population-specific recombination estimates and for detecting possible population substructures. It would also be interesting to carry out analyses between different age groups within populations.

3.4.7 Differences between 2008 and 2009

In the Edinburgh trial, timing of bud flush was measured again in 2009 at the beginning of the third growing season, and although ANOVA indicated significant among-population differences, the observed patterns were not associated with any environmental factors. In 2009, bud flush started and finished somewhat later than in 2008, resulting in bigger population means than in the previous year. However, the amount of within-population variation was not associated with site altitude.

It is possible that these patterns are due to the influence of the environment on the expression of complex traits: the same genetic makeup can be expressed differently in contrasting environments. Although the seedlings remained in the same glasshouse during this experiment, they might have been exposed to different temperatures in

2008 and 2009. During the winter of 2008/2009, the seedlings were protected from frost which might have influenced the trait's expression in the following spring.

For example, Campbell and Sorensen (1978) found that differences in timing of bud flush among coastal and inland Douglas-fir populations from western Washington and Oregon were largest in a warm soil-warm air treatment, while in a cool soil-cool air treatment, no association with distance from the sea was observed. Instead, altitude at the populations' origin became a significant factor. In an experiment by Leinonen (1996), an extended chilling period was found to minimise the differences in the high temperature requirement among North European and Scottish Scots pine populations, but the Scottish seedlings in this trial might have been subjected to an unnatural treatment because the experiment took place under natural outdoor conditions in Finland. These interactions can result in differing associations with environmental factors among different growing conditions (Falkenhagen 1979 and references therein) and therefore, phenotypic data from a single environment must be cautiously interpreted (Campbell 1986). In the future, it would be beneficial to continue scoring timing of bud flush in the same Scots pine seedlings as in 2008 and 2009 to better understand how the environment influences the trait's expression. Measurements could also be carried out outdoors where temperature accumulation is slower and seedlings are exposed to more natural temperatures.

3.5 Conclusions

This study examined variation in timing of bud flush among 21 native pinewoods in Scotland grown in two common-garden trials. The studied populations were from climatically diverse conditions and represented steep gradients for instance in the length of the growing season and winter temperatures. Significant associations between population means and environmental variables suggest that populations from colder areas with shorter growing seasons have an earlier bud flush, but a bigger proportion of the total variation was found among families within populations. Interestingly, the populations also varied in the level of variation in this trait, and

populations from high-altitude locations were characterised by the highest *CV*s, both on a population and family level. Analyses of temporal climate variation indicate that these populations also experience more environmental fluctuations, which might contribute to the trait variation in a species with different susceptibilities to climate-related mortality at different ages. Determining the relative contribution of fluctuating environmental conditions to the maintenance of adaptive variation - or proving its significance - is challenging in samples collected from *in situ* specimens of long-lived trees as the significance of such varying selection and also gene flow can vary temporally and spatially among populations. If temporal climate variation is important, one could expect to see similar differences in the variability of other quantitative traits. However, similar trends were not observed in 2009.

For studies on the maintenance of quantitative genetic variation in natural populations, forest trees can be considered difficult study objects due to their longevity and lack of experimental control over factors that might influence genetic variability in the course of their long life cycle. However, studying effects of temporally and spatially varying environments and causal relationships has proven challenging even in controlled laboratory populations of Drosophila (Mackay 1981; Roff 1997). Compared to short-lived model organisms like Arabidopsis and Drosophila, many tree species offer an intriguing platform for examining the potential role of environmental fluctuations in maintaining genetic diversity due to their wide ranges that differ not only spatially, but also in temporal variability, and to their life-history characteristics (e.g. decreasing susceptibility to climate-related factors at later developmental stages). While long-term climate data might be difficult to obtain for sparsely populated areas and for specific populations, trees offer the facility to trace back past climates in the form of tree rings (e.g. Hughes, Schweingruber et al. 1984). Many factors can contribute to patterns of genetic variation in natural populations, and understanding how the influence of these factors varies among populations is essential for sound interpretations of the data. In Scotland, many aspects of native pinewood biology remain unexplored, and more interdisciplinary research is needed.

In the future, to better understand how pine has adapted to its home environments and in order to provide a solid scientific background on which to base its management and conservation guidelines, it would be important to examine variation in other quantitative traits as well, and to study how populations respond when being transferred to a climate that differs from their home site conditions. Further examination of the potential role of temporally heterogeneous environments in maintaining quantitative trait variation in Scots pine would benefit from choosing a subset of populations representing a gradient in temporal climate variability and from more intensive within-population sampling. Patterns of variation could also be assessed in populations that were not included in our sample.

4. Fast phenotyping using chlorophyll fluorescence detects drought response in a common-garden trial of five native Scots pine (*Pinus sylvestris* L.) populations in Scotland

4.1 Abstract

Local adaptation is a common characteristic of forest trees, but it is not known whether the remaining native populations of Scots pine (*Pinus sylvestris* L.) in Scotland are adapted to environmental conditions at their home sites. Consequently, seed transfer guidelines for the species are based on selectively neutral molecular markers. Our aim was to test 20 open-pollinated families from five Scots pine populations covering a marked rainfall gradient to look for evidence of adaptive differentiation in their response to droughting. We measured water deficit and chlorophyll fluorescence in needles as well as plant mortality. Droughting resulted in an increase in water deficit which was reflected in reduction in parameters estimating the maximum quantum yield of primary photochemistry (F_v/F_m) and vitality (PI_{ABS}). Greater rates of mortality were observed in families with higher water deficit. Only PI_{ABS} showed significant among-population differentiation. Annual rainfall at home location was not associated with drought response, but families from sites with higher moisture deficit showed lower reduction in photochemical capacity, suggesting environment-driven adaptive divergence among sites and that a revision of marker-based seed zones in Scotland may be needed. We also found that chlorophyll fluorescence can be used as an efficient fast phenotyping tool in studies with large sample sizes.

4.2 Introduction

Many plant species occur over heterogeneous environments that impose contrasting selective pressures across their distribution. As a result, different genotypes are often favoured at different sites, leading to genetic differentiation among the populations (Linhart and Grant 1996). Local adaptation is considered to have taken place when individuals have highest fitness at their home site and if their performance is reduced when transferred to other environments (Kawecki and Ebert 2004). Trees often have wide geographic ranges covering steep environmental gradients and genetic differentiation in response to such variation among sample origins has been shown in many common-garden trials where associations between environmental and phenotypic variation have been found (reviewed in Howe, Aitken et al. 2003; Savolainen, Pyhäjärvi et al. 2007; Aitken, Yeaman et al. 2008). An understanding of how these patterns arise is essential for both forestry and conservation purposes: planting seedlings too far, either ecologically or geographically, from their home site often leads to increased mortality or poor performance (White, Adams et al. 2007), which can have substantial ecological, economic and practical consequences in a sector with often limited resources.

Scots pine (*Pinus sylvestris* L.) has a wide distribution, covering much of Eurasia from the Atlantic coast of Western Europe to Fennoscandia and eastern parts of Russia (Critchfield and Little 1966). In Scotland, 84 native pinewoods are recognized and their size varies from less than one to over 2,000 ha (Mason, Hampson et al. 2004). According to the most recent estimate, the total native pinewood area is approximately 18,000 ha (Jones 1999), only a small percentage of its postglacial maximum. In spite of this relatively small geographic area, environmental variation among pinewoods is significant (Mason, Hampson et al. 2004; Salmela, Cavers et al. 2010). Adaptations to local environments have been described in Scots pine in other parts of its range, for example in timing of growth (Sarvas 1962; Oleksyn, Tjoelker et al. 1992; Hurme, Repo et al. 1997) and cold tolerance (Hurme, Repo et al. 1997; Andersson and Fedorkov 2004), but little is known regarding the patterns of adaptive variation in Scotland and to date, only small-scale studies have been performed

(Perks and McKay 1997; Perks and Ennos 1999). Numerous projects in Scotland have been initiated to expand old and establish new pinewoods, and to guide seed transfers, the Forestry Commission developed guidelines in the late 1980s (McIntosh 2006). However, delineation of seed zones was based on earlier molecular marker studies which showed high levels of diversity and low levels of among-population differentiation (Forrest 1980; Forrest 1982; Kinloch, Westfall et al. 1986). Selectively neutral markers often fail to reflect adaptive differentiation (Merilä and Crnokrak 2001; McKay and Latta 2002), and given the extensive environmental variation within Scotland, it is unlikely that seed zones based on such markers will reflect patterns of adaptive variation among native pinewoods (Ennos, Worrell et al. 1998). To ensure the success of pinewood expansion and plantation of well-adapted woodlands, patterns of adaptive variation in quantitative traits in Scots pine should be assessed so that the risk of planting maladapted seedlings is minimised.

One of the steepest environmental gradients in Scotland is in annual rainfall and adaptation to this factor may be reflected in response to drought or waterlogging. Current climate change models predict that both drought in summer and waterlogging in winter will become more common in Scotland (Ray 2008), and if populations are locally adapted, they will differ in their response. Although phenotypic plasticity is a characteristic of many tree species (Mátyás 1996; Hamrick 2004), prolonged and more frequent extreme weather events could have serious consequences for forest trees and their ecosystems. Stress response can also vary among different age classes within species. For instance in two Mediterranean oak species, seedlings had lower water-use efficiency than adult trees (Mediavilla and Escudero 2004).

In response to drought, stomatal closure, decline in photosynthesis and, eventually, a growth reduction, are often observed (Pallardy 2008). However, measuring such events in a large number of samples can be challenging, time-consuming and expensive. Effects of stress on the photosynthetic machinery can be assessed, e.g., by measuring chlorophyll (*a*) fluorescence. This is a commonly used method which allows a quick evaluation of processes occurring at photosystem II (PSII), the site for

the light-driven part of photosynthesis (e.g. Baker and Rosenqvist 2004). Chlorophyll molecules in the thylakoid membranes absorb light energy for photosynthesis, part of which is also lost as heat or as fluorescence. Reduction in the energy consumption of one of these factors results in an increase in the other two, i.e., if photosynthesis is not able to function normally, more energy will be released as fluorescence (or heat). Such emissions can be recorded using portable fluorometers suitable for scoring many samples within a relatively short period of time. The ratio of two fluorescence parameters, F_v/F_m, has become a commonly used stress indicator and is close to 0.8 in healthy plants across various genera (Björkman and Demmig 1987). Also, good correlation between the state of the PSII and CO_2 fixation has been reported in controlled conditions (Genty, Briantais et al. 1989), but under natural conditions the association can be more complex (Maxwell and Johnson 2000). Thylakoid membranes of PSII are very sensitive to environmental stimuli (Bolhàr-Nordenkampf and Öquist 1993), and F_v/F_m has been reported to decline in response to various types of stress (Bolhàr-Nordenkampf and Öquist 1993; Mohammed, Binder et al. 1995; Maxwell and Johnson 2000). However, numerous studies have found it to be less sensitive than the less often used performance (or vitality) index PI_{ABS} (e.g. Force, Critchley et al. 2003; van Heerden, Merope et al. 2003; Oukarroum, Madidi et al. 2007) which takes into account not only F_v/F_m but also additional PSII features (Strasser, Srivastava et al. 1995, see Materials and Methods), possibly making it more responsive to stress treatments.

Chlorophyll fluorescence studies have revealed variation in drought or cold tolerance among genotypes of crops (Baker and Rosenqvist 2004) and tree species (Mohammed, Binder et al. 1995), however despite its ease of use, the method has not been extensively applied to studies where the interest is to partition natural variation into among and within-population components. The genetic basis of photosynthetic variation in trees has been previously demonstrated with variation found among families (Bigras 2000; Marshall, Rehfeldt et al. 2001; Koehn, Roberds et al. 2003; Bigras 2005), but to our knowledge only a few multi-population studies have been published. For instance, Parker, Rodriguez et al. (2003) used chlorophyll fluorescence to assess cold tolerance differences in 588 plants from 98 families in ten

populations of *Verbascum thapsus* L., an invasive weed, and more recently, the method was used to study drought tolerance among five populations of *Pinus canariensis* Chr. Sm. Ex DC (López, Rodríguez-Calcerrada et al. 2009). Both of these studies used F_v/F_m only. Our aim was to use chlorophyll fluorescence (F_v/F_m and PI_{ABS}) in a large sample of seedlings growing under common-garden conditions to test whether 1) native Scots pine populations in Scotland differed in their response to drought, 2) observed patterns of variation were linked to climatic differences between seedling origins, and 3) the method was a reliable and informative way of assessing drought effects in a sample of approximately 400 seedlings.

4.3 Materials and Methods

4.3.1 Study populations

Five native Scots pine populations were chosen for the study (figure 4.1). These populations represented the full extent of the annual rainfall gradient within Scotland, with site values varying from approximately 800 mm in the east to over 2,500 mm in the west (table 4.1). Estimates of average (1961-1990) growing season length and rainfall of the locations occupied by the populations were obtained from the UK Met Office gridded (5 × 5 km) datasets (Perry and Hollis 2005). Within each population, four mother trees were chosen at different altitudes and Ecological Site Classification (Pyatt, Ray et al. 2001) was used to estimate accumulated temperature (AT, accumulated day-degrees above a growth threshold, +5°C) and moisture deficit (MD, a measure of the dryness of the growing season) at each site. The model used in the estimation of AT assumes that AT decreases with increasing elevation, latitude, and longitude, while MD is calculated as the difference between monthly evaporation and monthly rainfall assuming that it decreases with elevation and latitude and increases with longitude. Evaporation estimates used by Ecological Site Classification are for 40 × 40 km grids and originate from the Meteorological Office

Rainfall and Evaporation Calculation System MORECS (Thompson, Barrie et al. 1982). Peat depth (PD) at each site was recorded during seed sampling.

4.3.2 Sampling

Open-pollinated seeds from four mother trees from each of five populations were sampled in winter 2007 and sown on trays (75:25 compost type John Innes 1: sand) in summer 2007. After germination seedlings were transferred to pots of size 0.62 l (diameter 11 cm, depth 9.6 cm) and kept under common-garden glasshouse and natural light conditions (glasshouse was shaded to avoid excess light) with watering applied 2-3 times per week during the growing season, depending on the weather conditions. Seedlings were potted up in spring 2008 (compost changed to John Innes 3) to pots of size $11 \times 11 \times 12$ cm (length $\times$ width $\times$ depth). The droughting trial was divided into 20 blocks of 20 seedlings each (i.e. one family member per block), and the order of families within blocks was randomized. For the drought study, ten treatment and ten control blocks were chosen. The drought treatment was applied between June 4 and July 9 2009 (droughted blocks were not given any water). Control samples were always watered one day before the measurements. Following the drought treatment all seedlings were watered normally. Height of the seedlings was measured weekly in June and total height reached was used in further analyses.
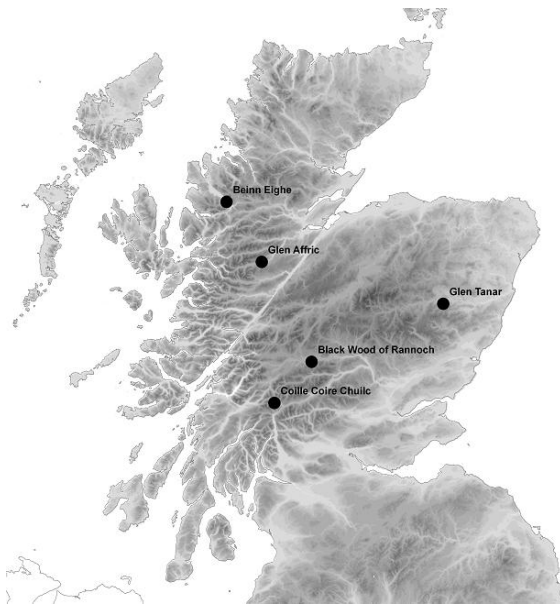
Figure 4.1 Locations of the sampled populations.

Table 4.1 Environmental data for populations and sites. Columns are pinewood size (PS) according to Mason, Hampson et al. (2004), growing season length (GSL), annual precipitation (AP), family (site) name, altitude, aspect (AS), accumulated temperature (AT), moisture deficit (MD), peat depth (PD), and average height of the seedlings in the family (AH).

Population	PS (ha)	GSL (days)	AP (mm)	Family	Altitude (m)	AS	AT (°C)	MD (mm)	PD (cm)	AH (cm)
Glen Tanar (GT)	1564	231	785	GT1851	289	NW	975	76	48	26.9
				GT1856	422	N	810	42	25	23.8
				GT1858	345	NW	906	62	32	25
				GT1860	330	NW	924	66	50	22.9
Black Wood of Rannoch (BW)	1011	252	1159	BW1822	307	N	990	57	50	27.4
				BW1825	257	S	1054	69	27	18.1
				BW1828	250	N	1063	71	28	21.2
				BW1830	285	N	1082	62	130	28.2
Glen Affric (GA)	1532	204	1685	GA1892	205	flat	1073	71	140	22
				GA1893	274	NW	990	53	40	22.6
				GA1897	274	NW	989	53	50	20.7
				GA1900	270	SE	994	54	35	27
Beinn Eighe (BE)	182	279	2411	BE21	59	NE	1210	103	70	26.7
				BE23	91	NE	1173	94	40	24.6
				BE26	83	NE	1183	97	45	21.9
				BE30	17	NE	1259	113	125	25.7
Coille Coire Chuilc (CCC)	67	223	2904	CCC1801	298	S	1024	56	50	24.8
				CCC1806	222	SE	1125	75	150+	22.8
				CCC1807	237	NE	1105	71	38	25.8
				CCC1809	269	S	1062	63	40	23.7

4.3.3 Drought stress

Water deficit

To estimate the effects of droughting on the seedlings' water status, water deficit (WD) was measured in 100 control and 100 droughted seedlings (five populations, four families/population, five seedlings/family/treatment) on two occasions (June 8 and 29). Three randomly chosen current-year needles were sampled per plant, and their fresh weight (FW) was measured. After this the needles were kept in deionized water overnight, and their weight at full turgor was measured (TW). Finally the needles were placed in an oven at ~75 °C for 3 days, after which their dry weight was measured (DW). Water deficit was calculated as WD=(TW-FW)/(TW-DW).

Chlorophyll fluorescence

Chlorophyll fluorescence was measured in 10 seedlings/family/treatment on three occasions (June 9-10, June 23-24, and June 30-July 1). The measurements were taken in the glasshouse over two days between 8.00 am and noon, and population and family means were calculated over this time period. Control and treatment blocks were scored alternately so that measurements for each population and family would be spread over the two days (five control and five treatment blocks per day). Two current-year needles from the opposite sides of the main stem and separate fascicles were used for the measurement (in some cases branches rather than the main stem were sampled), and their mean was used as the fluorescence value per plant. Needles were removed from the seedling, and clips were placed on them so that the flat (adaxial) side of the needle would be measured. Removal of leaves has been shown to have little effect on the recorded variables (Mohammed, Binder et al. 1995).

Prior to scoring, the needles were dark-adapted for approximately 20 minutes. After the dark adaptation, a Handy PEA portable fluorescence measurement system (Hansatech Instruments Ltd, Norfolk, UK) was used to give a saturating light pulse (intensity 2500 μM) resulting in fluorescence rising to its minimal level (F_0). At this stage all PSII reaction centres are open (Q_A, a primary electron transporter, in an oxidised state). After this, fluorescence quickly rises to its maximal level (F_m), a point where all electron transporters are reduced and reaction centres closed. Variable fluorescence (F_v) is the difference between F_m and F_0, and the maximum quantum yield of primary photochemistry at PSII can be expressed as F_v/F_m. We also recorded size of the area between F_m and F_0 (reflects the pool size of active electron transporters).

In addition, fluorescence values at five time points (50 microseconds, 100 microseconds, 300 microseconds, 2 and 30 milliseconds) between F_0 and F_m were recorded on June 23-24 and June 30-July 1. Using the JIP-test developed by Strasser, Srivastava et al. (1995), these fluorescence measurements can be used to estimate more detailed biophysical properties of PSII such as fluxes for absorption (ABS), energy trapping (TR), excess energy dissipation (DI) and electron transport further than Q_A (ET) per reaction centre (RC) and per cross-section of a sample (CS). Also, the formulae allow the estimation of the number of active RCs per cross section of a sample at the F_0 and F_m stages ($RC/CS_{0/m}$). Using the JIP parameters, F_v/F_m can also be expressed as $\varphi P_0 = TR_0/ABS$, i.e. the probability that an absorbed photon is trapped by a RC, reducing Q_A. ψ_0 is the probability that a trapped electron enters the electron transport chain and that Q_A is reoxidised, i.e. ET_0/TR_0, and quantum yield of energy dissipation (DI_0/ABS) can be estimated as $\varphi D_0 = 1 - \varphi P_0$. The performance index PI_{ABS} can be calculated using the derived parameters and the formula $PI_{ABS} = (RC/ABS) \times (\varphi P_0/(1 - \varphi P_0)) \times (\psi_0/(1 - \psi_0))$, where RC/ABS measures density of RCs per chlorophyll (for its derivation from the JIP-parameters, see e.g. Force, Critchley et al. 2003).

Visual damage to seedlings (mortality)

The droughted blocks were rewatered normally following the end of the drought stress. Nine weeks after the start of rewatering the proportion of fully brown (dead) seedlings was scored in each population and family.

4.3.4 Analysis

On each measurement date, a factorial analysis of variance (ANOVA) was used to test for the statistical significance of different factors. Due to deficiencies in glasshouse cover, a small number of seedlings did not receive complete droughting during treatment and these were excluded from subsequent analysis. Treatment and population were considered fixed factors and families within populations, interactions (treatment $\times$ population, treatment $\times$ family within population) and blocks within treatments were random. To test whether seedling size accounted for any of the variation in drought response, seedling height was included as a covariate. F_v/F_m ratios were transformed prior to analysis using the formula $F/(1-F)$, where $F=F_v/F_m$, to fit model assumptions better. Power transformation was used for PI_{ABS} ($PI_{ABS(sample)}^{0.50}$).

A correlation analysis was used to test for associations between the three response measures (WD, chlorophyll fluorescence, and mortality). Regression analysis was used to examine relationships between annual rainfall at site of origin and population means of WD, F_v/F_m and PI_{ABS} in the drought treatment on June 29 (WD) or June 23-24 (F_v/F_m, PI_{ABS}), and between site MD and PD and family means of WD, F_v/F_m, and PI_{ABS}. Ratios were transformed into continuous variables prior to analyses using the formula $X/(1-X)$, where X=measurement/population mean/family mean.

A chi-square (X^2) test was used to test for differences in proportion of fully brown seedlings among populations. The proportions of dead seedlings in each family were compared to family means of WD, F_v/F_m, and PI_{ABS}.

Analyses were carried out using GenStat 10.2.0.175.

4.4 Results

4.4.1 Water deficit (WD)

On June 8, shortly after the beginning of the drought treatment, population means for WD varied between 0.28 and 0.30. No significant differences due to treatment, populations, families or their interactions were observed (table 4.2a). Four weeks later, on June 29, the means between the two treatments varied significantly (table 4.2b): the means of the control and the drought treatments were 0.27 and 0.51, respectively, and within the control samples the means varied between 0.24 and 0.30, while in the drought treatment WD varied between 0.47 and 0.56 (figure 4.2). Highest WD was measured in GT and CCC (0.53 and 0.56, respectively), and was lowest in BW and GA (0.47 and 0.48, respectively). The mean in BE was 0.52. Pooling samples across the treatments, no significant among-population differentiation was found, while families within populations were significantly different. Interaction terms were also significant. Seedling height was significantly associated with WD; however, the same components remained significant when height was removed from the analysis.

Associations with environmental factors

Population means were not associated with annual rainfall at site of origin (p=0.36, R^2=0.044). MD and PD at the site of origin explained only 0.30% of the variation in the family means (p=0.38).
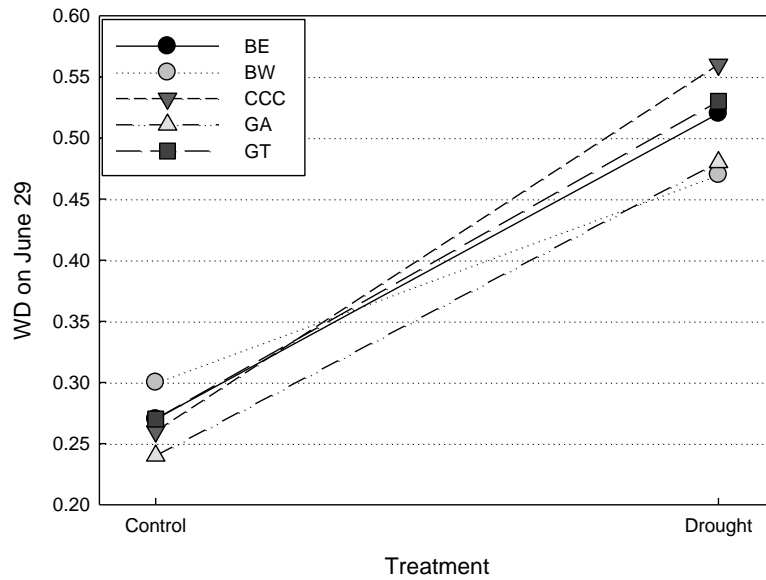
Figure 4.2 Interaction plot of population means of WD on June 29.

Table 4.2 ANOVA tables with mean squares for WD on a) June 9 and b) 29. ns=P>0.05; *=P<0.05; **=P<0.01, ***=P<0.001.

a) Date: June 8	df	MS
Treatment	1	0.000441 ns
Population	4	0.00385 ns
Families within population	15	0.00517 ns
Treatment x population	4	0.00137 ns
Treatment x family within population	15	0.00656 ns
Blocks within treatments	8	0.02057 **
Covariate (height)	1	0.00122 ns
Residual	135	0.00647

b) Date: June 29	df	MS
Treatment	1	2.811 ***
Population	4	0.0157 ns
Families within population	15	0.0134 *
Treatment x population	4	0.0282 **
Treatment x family within population	15	0.0194 ***
Blocks within treatments	8	0.0280 ***
Covariate (height)	1	0.0540 **
Residual	135	0.00682

Covariate coefficient (s.e.): 0.0038 (0.00136)

4.4.2 Chlorophyll fluorescence

F_v/F_m

On June 9-10, about one week after the onset of the treatment, the droughted and control seedlings had very similar F_v/F_m (0.73 and 0.74, respectively; figure 4.3a). The difference was not statistically significant (table 4.4a). Within the two treatments the population means were very similar and varied between 0.72 and 0.74 (table 4.3). Of all factors, only blocks within treatments was statistically significant. F_v/F_m values recorded closer to noon tended to be lower than those recorded early in the morning (on June 9, $y=0.76-0.021$(hours from the start), $p<0.0001$, $R^2=0.14$). Similar patterns were observed on June 10 (data not shown).

Two weeks later on June 23-24, a significantly lower value of F_v/F_m (0.61) was observed in the drought treatment compared to the control seedlings (0.77; figure 4.3a and table 4.4b). Within the drought treatment, lowest means were recorded in GT (0.56) and CCC (0.58) and the highest in BE (0.67). However, the population means across the treatments were not significantly different. Families within populations across the treatments did not contribute significantly to the total variance, but the treatment affected families differently.

On June 30-July 1, statistically significant factors were treatment, interaction of treatment with families within populations, and blocks within treatment. The mean of the droughted seedlings had dropped to 0.42, compared to 0.79 recorded in the control seedlings (figure 3a). The lowest means among the droughted samples were observed in GT (0.35) and CCC (0.40), and the highest in GA (0.47), BE and BW (0.46 in both; table 4.3).

Main effects of the factors were not significantly influenced by the height of the seedlings (table 4.4).

PI_{ABS}

On June 23-24, the mean of PI_{ABS} was 1.57 in the control and 0.71 in the drought samples (figure 4.4), and the index mean varied between 1.41 and 1.75 (GT and BE) in the control samples and between 0.55 and 0.95 (GT and BE) among the droughted samples (table 4.3). Significant differences were observed among treatments, populations across the treatments and blocks within treatments (table 4.4b). Across the two treatments, a strong and positive linear correlation (r=0.95, p<0.0001) was observed between $ln(PI_{ABS})$ and $ln((F_v/F_m)/(1-F_v/F_m))$.

On June 30-July 1, we observed significant differences between treatments and blocks within treatments (table 4.4c). The mean of PI_{ABS} in the control treatment had increased to 1.95 while in the drought treatment the mean had dropped to 0.23 (figure 4.4). Within the control samples, population means varied between 1.85 (CCC) and 2.08 (BE) and between 0.14 (GT) and 0.23 (BE, BW) in the droughted samples (table 4.3).

Main effects of the factors were not significantly influenced by the height of the seedlings (table 4.4).

Effects of droughting on fluorescence parameters

When means in the two treatments were compared, a decrease in F_v/F_m was found to be due to both an increase in F_0 (+36%) and a decrease in F_m (-17%), resulting in lower F_v (-31%). The size of the area between F_m and F_0 also decreased (-42%). JIP parameters showed that TR_0/ABS (F_v/F_m) decreased by 22% and ET_0/TR_0 by 21%, while DI_0/TR increased by 76%. The number of reaction centres per cross-section of a sample (RC/CS) decreased by 18% (RC/CS_0, minimal fluorescence stage) or 39% (RC/CS_m, maximal fluorescence stage).

Associations with environmental factors and WD

A significant correlation was found between WD on June 29 and F_v/F_m in individual seedlings on June 23-24 ($r=-0.56$, $p<0.0001$, figure 4.5); a similar pattern was observed with PI_{ABS} ($r=-0.46$, $p<0.0001$). Annual rainfall did not explain any of the variation among population means of F_v/F_m or PI_{ABS} ($p=0.70$ and $p=0.79$, respectively). However, family means of F_v/F_m on June 23-24 were significantly associated with home site altitude ($\beta_0=2.335$, $\beta_1=-0.003025$, $p<0.001$, $R^2=44\%$) and MD ($\beta_0=0.411$, $\beta_1=0.017$, $p<0.001$ $R^2=46\%$, figure 4.6). There were no associations with PD. Similar trends were also found for PI_{ABS}: lower PI_{ABS} was observed at higher altitudes ($\beta_0= 1.049$, $\beta_1=-0.001437$, $p=0.005$, $R^2=33\%$) and at sites with lower MD ($\beta_0= 0.134$, $\beta_1=0.0081$, $p=0.004$, $R^2=34\%$). Sites at BE differed distinctly from other sites in altitude and MD (figure 4.6, table 4.1), but the associations remained significant even after removing BE values (for F_v/F_m and altitude, $\beta_0=2.506$, $\beta_1=-0.00357$, $p=0.026$, $R^2=26\%$); with MD, $\beta_0=0.304$, $\beta_1=0.019$, $p=0.030$, $R^2=24\%$).

Table 4.3 Population means and standard deviations of F_v/F_m on June 9-10, June 23-24, and June 30-July 1, and of PI_{ABS} on June 23-24 and June 30-July 1.

Population		F_v/F_m June 9-10		F_v/F_m June 23-24		F_v/F_m June 30-July 1		PI_{ABS} June 23-24		PI_{ABS} June 30-July 1	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control:	GT	0.74	0.04	0.78	0.04	0.80	0.02	1.41	0.61	1.89	0.78
	BW	0.73	0.04	0.77	0.04	0.79	0.03	1.64	0.92	1.92	0.88
	GA	0.73	0.05	0.77	0.04	0.79	0.03	1.61	1.00	2.03	0.97
	BE	0.74	0.04	0.78	0.04	0.79	0.03	1.75	0.81	2.08	0.90
	CCC	0.74	0.06	0.77	0.07	0.78	0.04	1.44	0.79	1.85	0.88
Drought:	GT	0.72	0.05	0.56	0.24	0.35	0.20	0.55	0.57	0.14	0.32
	BW	0.74	0.04	0.64	0.18	0.46	0.22	0.83	0.78	0.23	0.38
	GA	0.73	0.06	0.60	0.22	0.47	0.22	0.61	0.68	0.20	0.29
	BE	0.73	0.07	0.67	0.16	0.46	0.22	0.95	0.87	0.23	0.29
	CCC	0.74	0.06	0.58	0.21	0.40	0.23	0.58	0.62	0.16	0.24

Table 4.4 ANOVA tables with mean squares for F_v/F_m and PI_{ABS} on June a) 9-10, b) 23-24, and c) June 30-July 1. ns=P>0.05; *=P<0.05; **=P<0.01, ***=P<0.001.

a) June 9-10	df	F_v/F_m	PI_{ABS}
Treatment	1	0.487 ns	
Population	4	0.879 ns	
Families within population	15	0.404 ns	
Treatment x population	4	0.161 ns	Not recorded
Treatment x family within population	15	0.376 ns	
Blocks within treatments	18	2.891 ***	
Covariate (height)	1	0.036 ns	
Residual	323	0.287	

b) June 23-24	df	F_v/F_m	PI_{ABS}
Treatment	1	196.934 ***	25.203 ***
Population	4	1.647 ns	0.550 *
Families within population	15	0.878 ns	0.151 ns
Treatment x population	4	0.982 ns	0.0766 ns
Treatment x family within population	15	1.320 *	0.147 ns
Blocks within treatments	18	11.310 ***	1.168 ***
Covariate (height)	1	1.424 ns	0.0402 ns
Residual	324	0.732	0.103

c) June 30-July 1	df	F_v/F_m	PI_{ABS}
Treatment	1	805.247 ***	107.0930 ***
Population	4	0.988 ns	0.142 ns
Families within population	15	0.648 ns	0.125 ns
Treatment x population	4	0.966 ns	0.0276 ns
Treatment x family within population	15	1.0436 *	0.0594 ns
Blocks within treatments	18	3.133 ***	0.266 ***
Covariate (height)	1	0.00330 ns	0.177 ns
Residual	313	0.54	0.0781

4.4.3 Proportion of fully brown seedlings (mortality)

The proportion of fully brown seedlings in populations within the drought treatment varied between 0.47 in GA and 0.65 in CCC. There were no statistically significant differences in the frequency of surviving seedlings among the five populations (χ^2= 2.73, df=4, p=0.60). However, the proportion of brown seedlings was higher in families with higher WD on June 29 (r=0.54, p=0.014) and with lower F_v/F_m on June 23-24 (r=-0.56, p=0.011). A similar correlation was observed with F_v/F_m on June 30-July 1 (r=-0.53, p=0.017). Correlations with PI_{ABS} on June 23-24 or June 30-July 1 were similar in direction but not statistically significant (r=-0.34, p=0.14; r=-0.37, p=0.11, respectively).
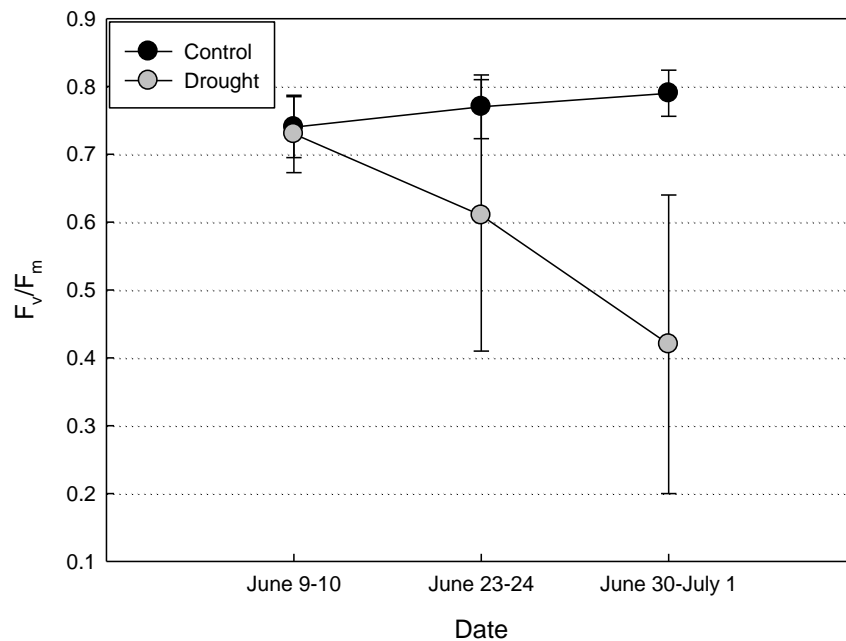
Figure 4.3 Control and drought treatment means and standard deviations of F_v/F_m between June 9-10 and June 30-July 1.
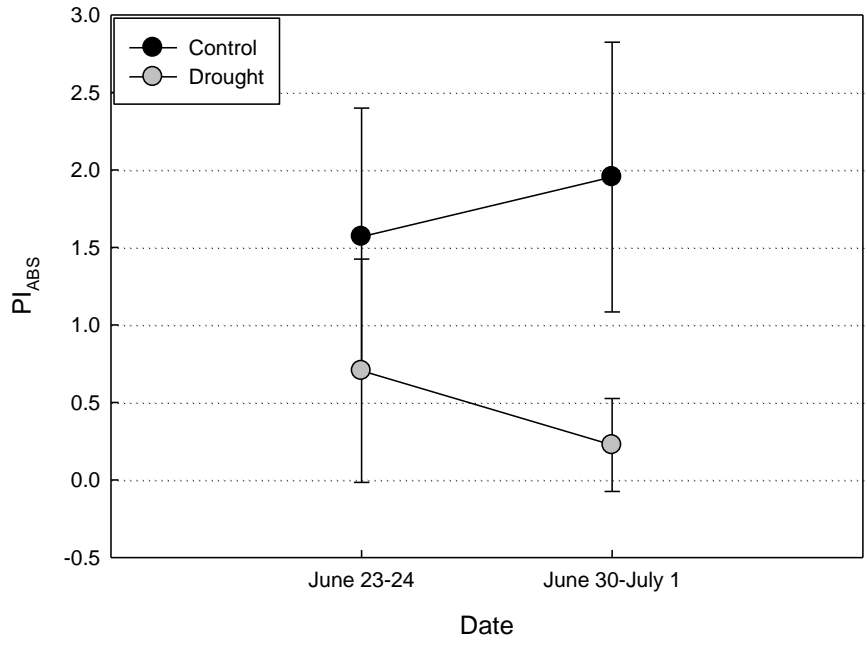
Figure 4.4 Control and drought treatment means and standard deviations of PI_{ABS} on June 23-24 and June 30-July 1.
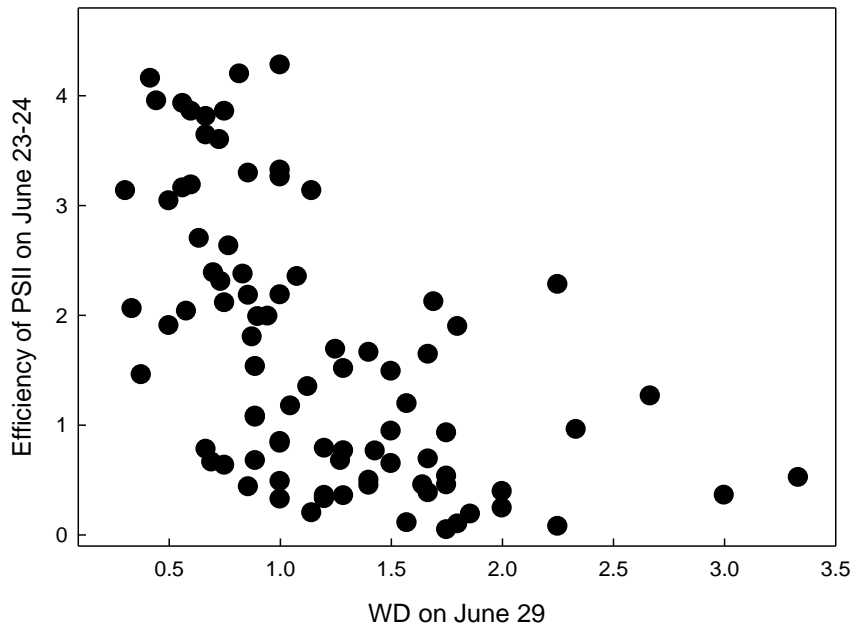
Figure 4.5 Relationship between WD on June 29 and efficiency of PSII within the drought treatment on June 23-24 (r=-0.56, p<0.0001). Efficiency of PSII=$F_v/F_m/(1-(F_v/F_m))$, and water deficit=WD/(1-WD).
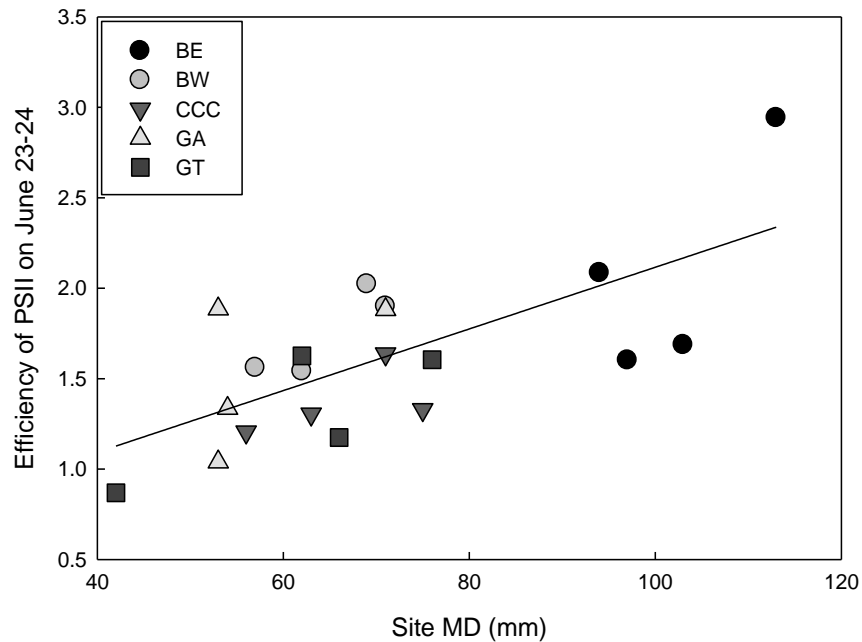
Figure 4.6 Relationship between site MD and family means of efficiency of PSII on June 23-24 (β_0=0.411, β_1=0.017, p=0.0007, R^2=0.46). Efficiency of PSII=$F_v/F_m/(1-(F_v/F_m))$.

4.5 Discussion

In this study we examined whether three-year old Scots pine seedlings from five populations and 20 families originating from areas with very different annual rainfall and subjected to drought stress showed evidence of adaptation to their home environments. We found that droughting led to WD in seedlings and that loss of water was reflected in lower efficiency of PSII (reduction in F_v/F_m and PI_{ABS}). Also, higher WD and lower F_v/F_m were associated with increased mortality. However, we did not find associations between annual rainfall and population means of the fluorescence parameters. Instead, family means were linked to MD, an estimate of the dryness of the growing season that not only takes into account rainfall, but also evaporation. Compared to the other methods used, chlorophyll fluorescence was the most rapid and sensitive method for assessing effects of droughting, allowing 400 measurements in a matter of a few hours.

4.5.1 Response to drought

The photosynthetic responses of plants to various types of treatments have been extensively studied and the photosynthetic process has been shown to be very sensitive to external stimuli (Maxwell and Johnson 2000; Baker and Rosenqvist 2004). WD usually leads to stomatal closure, a decrease in mesophyll conductance and slower rates of CO_2 assimilation (Pallardy 2008) , and if the plant is still absorbing light energy, photosynthetic processes must be down-regulated to minimize imbalance between the supply of reducing power through PSII and its use in the dark reactions (Krause 1988). Chlorophyll fluorescence is a quick, easy and informative way to study stress effects on the sensitive PSII, and while stomatal closure on its own does not lead to a decrease in the efficiency of the photosynthetic machinery, it will be followed by a decrease in the use of products of the light reactions in CO_2 assimilation which could be reflected in fluorescence parameters (Baker and Rosenqvist 2004). We did not observe any treatment effects in F_v/F_m after about one week of droughting, and the possible response in PI_{ABS} was not recorded at that stage. However, PI_{ABS} has been shown to be more sensitive to stress than F_v/F_m (van Heerden, Merope et al. 2003; Christen, Schönmann et al. 2007; van Heerden, Swanepoel et al. 2007) and it is possible that it would have shown a response earlier than F_v/F_m. The sensitivity of PI_{ABS} was evident in our data on June 23-24 when it showed bigger and significant differences among populations: population means for F_v/F_m in the drought treatment were 72-86% of those observed in the control treatment, while the respective values in PI_{ABS} were only 37-54%. This difference can be attributed to the more complex calculation of PI_{ABS}: while F_v/F_m considers only the difference between F_0 and F_m, PI_{ABS} is estimated from three independent expressions related to PSII light-harvesting antenna properties, primary photochemistry (F_v/F_m) or further electron transport capability that are calculated from additional fluorescence points between F_m and F_0. This means PI_{ABS} can respond to changes in any of these three components (Oukarroum, Madidi et al. 2007). However, we found that the correlation between the two measures was strong and that the ranking of the populations was the same in both cases, indicating that both parameters measure the same process, only with different sensitivity. Also,

mortality (estimated as the proportion of fully brown seedlings) was found to be associated with higher WD and lower F_v/F_m, but many seedlings recovered from the stress following rewatering, as evidenced by the presence of healthy-looking green needles in early autumn. However, in many cases stems appeared permanently damaged and green needles were restricted to certain parts of seedlings, suggesting that droughting might have severely damaged their growth form.

Examining individual components of F_v/F_m and PI_{ABS} can shed light on how PSII and its components are functioning under stress. Comparisons between the means of individual fluorescence parameters in the control and drought treatments showed that the decrease in F_v/F_m was due to both an F_0 increase and an F_m decrease. This is in contrast to *Picea glauca* (Moench) Voss (Bigras 2005) or *Pinus radiata* D. Don (Conroy, Smillie et al. 1986) in which a decline in F_m alone was observed, possibly indicating differences in drought tolerance among species occurring in environments with varying moisture conditions. Drought also reduced the pool size of available electron transporters. Increasing F_0 is thought to reflect RC damage at PSII (Krause and Weis 1984) while lower F_m is related to an increase in conversion of excess energy into heat (Müller, Li et al. 2001). Indeed, the number of active RCs at F_0 and F_m stages was lower in the drought treatment, and inactive RCs can function as protective sinks for excess energy which could also explain decline in F_m (Öquist, Chow et al. 1992). The release of excess energy was also reflected in the quantum yield of energy dissipation which increased by 76%. Droughting had a negative effect on further electron transport (ET_0/TR) which decreased in a similar fashion to F_v/F_m (TR_0/ABS).

4.5.2 Variation among populations and families

Surprisingly, despite significant variation in annual precipitation (~800-2,900 mm) among the study populations, we found no link between annual rainfall and population response to drought. Scots pine occurs in highly heterogeneous environments within Scotland, and such extensive environmental variation provides

ideal conditions for local adaptation to occur. The rainfall gradient within Scotland is very steep, and as a result it is possible that native pinewoods may respond differently to water stress. On June 29, the highest WDs were recorded in CCC and GT, the wettest and the driest population, respectively, in terms of annual rainfall. A similar pattern was seen in F_v/F_m as well as PI_{ABS}. Chlorophyll fluorescence studies attempting to link phenotypic variation to environmental variation among multiple populations are still rare. Testing for stress tolerance differences in a weedy species, Parker, Rodriguez et al. (2003) applied the method to a 10-population sample and found significant differences in freezing tolerance (estimated as F_v/F_m), but no significant associations between phenotypic and environmental variation, possibly due to life-history characteristics of herbaceous weed species. López, Rodríguez-Calcerrada et al. (2009) studied drought tolerance among five populations of *Pinus canariensis* (six seedlings/provenance in each of the three treatments) that differed in their annual rainfall. They found no significant differences in F_v/F_m, although it is possible that PI_{ABS} would have been a more informative parameter as it has been reported to change while F_v/F_m has remained relatively stable under stress (Oukarroum, Madidi et al. 2007). However, the method was successfully used to demonstrate adaptive divergence in cold tolerance among populations of *Quercus suber* L. (Aranda, Castro et al. 2005). It is unlikely that the pattern we observed is due to random genetic drift as a result of heavy exploitation of pinewood resources during pine's history in Scotland as neutral molecular marker data do not show any significant reductions in the level of diversity compared to more continuous continental populations (Forrest 1980; Kinloch, Westfall et al. 1986; Provan, Soranzo et al. 1998; Wachowiak, Salmela et al. 2011). Phenotypic divergence among Scottish pinewoods has been reported before (Perks and McKay 1997; Perks and Ennos 1999), but only a few provenances were sampled and no clear environmental trends could be established.

Although the observed population-level patterns appeared to be independent of the rainfall gradient, significant associations were found between altitude at origin, site MD estimate, and both F_v/F_m and PI_{ABS}. The smallest response to stress was observed in seedlings from BE in north-western Scotland where four low-altitude, high-MD

sites were sampled. All other sites were at altitudes higher than 205 m and had noticeably lower site MDs. However, although somewhat weaker, the association remained significant even when BE was excluded from the analysis. MD is a measure that takes into account not only precipitation but also evaporation, and reflects the dryness of the growing season rather than that of the whole growing year (Pyatt, Ray et al. 2001). However, site-specific MD estimates are obtained through interpolation using rainfall and evaporation data from a wider geographic scale and therefore the precision of the estimates could not be verified. Nonetheless, associations with altitude are significant, suggesting that the factor causing the observed differences might vary along elevation in this set of data. Rainfall is often used as a proxy for site moisture conditions, and for example Cregg and Zhang (2001) found that Scots pine populations with lower annual rainfall along a longitudinal gradient of 100° had higher intrinsic water-use efficiency, estimated using carbon isotope discrimination, but survival under drought was not significantly different among the 12 populations sampled. Green (1964) has noted that 'effective wetness' is not determined by rainfall alone on the British Isles, and our results are in accordance with this statement. Soil is the primary source of water for plants, and therefore, soil characteristics also greatly influence water availability (Nilsen and Orcutt 1996). Factors playing a role include, e.g., topography, aspect of the site, soil texture, organic matter, stratification, salinity, and microbial activity. The sites we sampled vary in their altitude, continentality, aspect and slope which could complicate the use of only one general moisture variable. Peat depth was recorded at the home site of each family, but it was not significantly associated with any of the fluorescence variables or WD. However, only one measurement of peat depth was taken and this might not fully represent the characteristics of each site. As we only considered rainfall when choosing our study populations, we do not know if the MD gradient in our study is representative of the whole of Scotland. In future, it would be interesting to base sampling for drought-testing on variation in MD across native Scottish pinewoods to verify whether MD or rainfall is more significant in driving differentiation. Also, a better understanding of the causes of the observed variation in fluorescence parameters could be gained by studying morphological (e.g. needle

characteristics such as stomatal density or size) and biochemical variation among populations.

Several components of photosynthesis have been shown to vary among families of trees (Marshall, Rehfeldt et al. 2001; Koehn, Roberds et al. 2003), indicating that associated traits have a genetic basis. In our study, by late June, WD showed significant differences among families within populations, suggesting heritable variation in drought susceptibility within populations. Further evidence of such variation was seen in F_v/F_m which was differently affected among droughted families. It is important to understand how much variation there is in phenotypes of fragmented and small populations as natural selection can only act on heritable variation (additive genetic variation): the more such variation resides in a population, the more potential it has to adapt to changing selection pressures (e.g. Falconer and Mackay 1996). A larger number of families within populations would be required to study the heritable nature of the process in more detail. However, trees usually have high levels of heritable variation because of outcrossing and efficient gene flow among populations occurring in diverse environments (Howe, Aitken et al. 2003; Savolainen, Pyhäjärvi et al. 2007; Aitken, Yeaman et al. 2008).

4.5.3 Summary

In summary, we found that droughting caused WD in Scots pine seedlings, the effects of which were also seen in F_v/F_m, PI_{ABS}, and mortality. We found some evidence of adaptive divergence among populations and families, suggesting that severe historical woodland fragmentation and exploitation have not severely interfered with potential local adaptation. In view of plans to expand current native pinewood coverage, an understanding of Scots pine's adaptations to local conditions in Scotland is important and such data should be incorporated into management and conservation practices. However, although the observed patterns of phenotypic variation in response to droughting were found to be associated with an environmental variable, proving local adaptation requires some demonstration that

the trait confers higher fitness at the home site, such as by reciprocal transplant experiments. In accordance with earlier findings, we found that PI_{ABS} is a much more sensitive stress indicator than F_v/F_m; however, the two were strongly correlated and showed very similar environmental trends. We found that chlorophyll fluorescence can be an efficient tool even with large datasets and that in addition to studying differences among only a few genotypes, the method has potential to become a valuable and more regularly used tool in evolutionary biology for assessing treatment effects in studies of natural variation in plant populations. Such high-throughput phenotyping methods are in great demand in phenomics, the study of phenotypes and their co-evolution, a field that has received much less attention than for instance complex-trait genomics due to being considered expensive, time-consuming, and laborious (Houle 2010).

4.6 Acknowledgements

5. Seasonal patterns of photochemical capacity and spring phenology reveal genetic differentiation among eight native Scots pine (*Pinus sylvestris* L.) populations in Scotland

5.1. Abstract

Environment-driven genetic differentiation among populations is a common feature among forest trees, and an understanding of how populations have adapted to their home site conditions is essential for management and conservation practices. In Scotland, 84 native Scots pine (*Pinus sylvestris* L.) woodlands are recognized by Forestry Commission and they occupy highly diverse environments from the maritime west coast to continental sites in eastern Scotland. However, it is not known whether adaptations to local environments along sharp temperature and rainfall gradients have occurred in different populations and as a result, the seed transfer guidelines of the species are based on selectively neutral molecular markers. In this study, we used chlorophyll fluorescence to examine whether fourth-year seedlings from 32 open-pollinated families and eight populations from sites experiencing contrasting annual temperature regimes differed in their response to variation in natural outdoor temperatures between September 2009 and May 2010. In addition, we measured growth initiation in spring. Photochemical capacity at photosystem II (F_v/F_m) showed a distinct seasonal trend and remained at relatively high levels (~0.7) until December. Following a period of over two weeks with temperatures below or close to 0°C, it started decreasing towards its minimum values recorded in early March when population means varied between 0.35 and 0.45. By early May and along with rising temperatures, photochemical capacity had recovered to the same level as observed in early December. Populations were found to respond differently to the cold period starting in December, with the largest drop in photochemical capacity being observed in seedlings from the mild and maritime western Scotland. In March, the recovery of photochemical capacity was slowest in seedlings from the mildest and coolest sites. Evidence of adaptive genetic differentiation was also found

in spring phenology. Initiation of shoot elongation and needle flush were earlier in families and populations from cooler areas. These results suggest that adaptation to the spatially heterogeneous environment in Scotland has taken place in Scots pine and that in order to minimise the risk of planting maladapted seed stock, the patterns of environmental and adaptive genetic variation should be taken into account in the management of genetic resources in this species.

5.2 Introduction

When a species is found over a spatially heterogeneous environment, divergent selection among different parts of the range often results in a situation where populations become genetically differentiated and adapted to the conditions in their home environments (e.g. Linhart and Grant 1996; Kawecki and Ebert 2004). Many widespread tree species occur across sharp ecological gradients in temperature and moisture conditions (e.g. Petit and Hampe 2006) and have evolved significant genetic differentiation in quantitative traits among populations. These differences are evident when provenances are grown together under common-garden conditions and when seed is transferred to sites that differ environmentally from its home site (reviewed in Howe, Aitken et al. 2003; Savolainen, Pyhäjärvi et al. 2007; Aitken, Yeaman et al. 2008). Studying local adaptation in trees has a long history due to the economic importance of many species (Mátyás 1996), but understanding associations between environmental and phenotypic variation is also required for conservation management in species with mostly ecological importance (McKay, Christian et al. 2005).

Scotland represents a geographically separate, north-western tip of the wide pan-Eurasian distribution of Scots pine (*Pinus sylvestris* L.) (Critchfield and Little 1966). Currently 84 native pinewoods of variable size are recognized by the Great Britains's Forestry Commission (Jones 1999), which together constitute about 1% of the maximum postglacial coverage (e.g. Mason, Hampson et al. 2004). Despite significant historical population size decrease, most populations are still diverse at

selectively neutral molecular markers, with only a small proportion of the variation due to among-population differences (Forrest 1980; Kinloch, Westfall et al. 1986; Provan, Soranzo et al. 1998). Moreover, variation at several candidate genes in Scottish populations was similar to that in more continuous continental populations (Wachowiak, Salmela et al. 2011). However, little is currently known about patterns of adaptive variation in phenotypic traits among Scottish pinewoods. The performance of continental provenances in Scotland is worse than that of native provenances grown on the same site (Worrell 1992), but within Scotland only few small-scale studies have been done so far (Perks and McKay 1997; Perks and Ennos 1999) and it is not known how adaptive traits vary among populations as a result of possible genetic differentiation. Despite the relatively small area covered by native pinewoods (maximum distance between two pinewoods is less than 200 km and the total area of native woodland is approximately 18,000 ha), they occur over a range of altitudes and environmentally diverse conditions, with significant gradients in rainfall patterns and temperature features (Salmela, Cavers et al. 2010). Evidence for environment-driven genetic differentiation in adaptive traits among continental Scots pine populations has been found in previous common-garden studies where sampling has often been done on a much wider geographic scale (e.g. Sarvas 1962; Wright, Pauley et al. 1966; Steiner 1979; Oleksyn, Tjoelker et al. 1992; Hurme, Repo et al. 1997; Cregg and Zhang 2001; Andersson and Fedorkov 2004). Due to the lack of knowledge on patterns of adaptive variation in Scotland, the current seed transfer guidelines are based on earlier work using neutral molecular markers (Forrest 1980; Kinloch, Westfall et al. 1986), and divide Scotland into seven geographical zones of biochemical similarity. However, while being valuable tools for studies of population structure and gene flow, such markers often fail to reflect adaptive divergence in phenotype among different populations (Karhu, Hurme et al. 1996; McKay and Latta 2002). To ensure the conservation of adaptive genetic variation and the maintenance of evolutionary potential in Scottish populations in future, an extensive assessment of patterns of adaptive variation in relation to local environments is needed.

Seasonality characterises the natural habitats of temperate and boreal zone tree species, where environmental conditions are ideal for growth for a limited period of

time. Trees have adapted to such environmental variation by switching between periods of active growth and dormancy in response to changes in temperature or photoperiod (Howe, Aitken et al. 2003). Photosynthesis shows clear seasonal variation in relation to temperature conditions in northern conifers (Öquist and Huner 2003): in Scots pine growing in northern Sweden, photosynthetic activity declined along with decreasing autumn temperatures, was inhibited in the middle of winter due to freezing temperatures, and recovered in spring as temperatures started to rise (Ottander, Campbell et al. 1995). Similarly, the phenology of events such as growth initiation and cessation are also governed by environmental cues (Howe, Aitken et al. 2003). By timing growth to suit their environment, trees minimise the risk of frost injury at the beginning and the end of their growth periods and maximise their growth potential. Commonly trees from sites with shorter growing seasons respond to rising temperatures earlier in spring (Steiner 1979; Beuker 1994) and cease growing (Oleksyn, Tjoelker et al. 1992) and develop cold hardiness earlier in autumn (Hurme, Repo et al. 1997) when kept under common-garden conditions. Mismatches between growth phenology and environment can lead to poorer fitness or growth performance: in northern Sweden, transferring seedlings further north has been found to result in increased mortality, and while transfers south increase survival, the transferred trees grow less than the local population due to earlier cessation of growth (Persson and Ståhl 1990).

Initiation and cessation of growth can be easily scored in a large number of young seedlings and variation in these traits has been extensively studied in forest trees (Howe, Aitken et al. 2003; Savolainen, Pyhäjärvi et al. 2007; Aitken, Yeaman et al. 2008). How photosynthetic activity varies in different seasons among populations from diverse climates growing under common-garden conditions has not been examined in such great detail. Chlorophyll (*a*) fluorescence is an efficient and commonly used method allowing a quick evaluation of processes occurring at photosystem II (PSII), the site for the light-driven part of photosynthesis (e.g. Baker and Rosenqvist 2004). The ratio of two fluorescence parameters, F_v/F_m, is a measure of the maximum quantum yield of primary photochemistry at PSII and is close to 0.8 in healthy plants across various genera (Björkman and Demmig 1987). Also, a good

correlation between F_v/F_m and CO_2 fixation has been reported in controlled conditions (Genty, Briantais et al. 1989), but the relationship can be more complex in natural environments due to other competing processes (Maxwell and Johnson 2000). PSII is sensitive to environmental stimuli and responses to rising or decreasing temperatures are often reflected in an increase or a decline in F_v/F_m, respectively (Bolhàr-Nordenkampf and Öquist 1993). Chlorophyll fluorescence has been extensively used in plant physiology and to study stress tolerance among relatively small numbers of genotypes, but has yet to become a popular tool in evolutionary biology for assessing differences in photosynthetic activity in response to environmental signals among multiple populations, and only a few such studies have been published so far (Parker, Rodriguez et al. 2003; Aranda, Castro et al. 2005; López, Rodríguez-Calcerrada et al. 2009).

In this study, our aim was to examine seasonal patterns of photochemical capacity at PSII (estimated as F_v/F_m) under natural climatic conditions in an outdoor common-garden trial of eight native Scots pine populations in Scotland covering a steep gradient in growing season length and annual temperature conditions. We tested the hypothesis that, if genetically differentiated and adapted to the annual temperature regime at their home site, then populations would vary in their response to changing temperatures, and that the patterns of variation would be associated with the environmental characteristics at their home sites. We also recorded timing of growth initiation in spring to test whether phenological differences among populations reflected potential adaptation to local conditions.

5.3 Materials and methods

5.3.1 Study populations

Eight native Scots pine populations were chosen for the study (figure 5.1). The locations these populations occupy represent a gradient in the temperature-driven growing season length within Scotland (table 5.1). UK Met Office climate data were used to estimate average (1961-1990) growing season length (GSL), mean February and July temperatures and annual number of air and ground frost days (Perry and Hollis 2005) at each location. The climate data for the UK are divided into 5×5 km grids and are partly based on interpolation, especially in parts of the Highlands where weather stations are not equally distributed across the landscape and different altitudes. Therefore the climatic variables should only be considered as proxies. Within each population, four mother trees growing at different altitudes (sites) were chosen, and Ecological Site Classification (Pyatt, Ray et al. 2001) was used to estimate annual accumulated temperature at these sites (AT, accumulated day-degrees above a growth threshold, +5°C). The model used in the estimation of AT assumes that AT decreases with elevation, latitude and longitude.
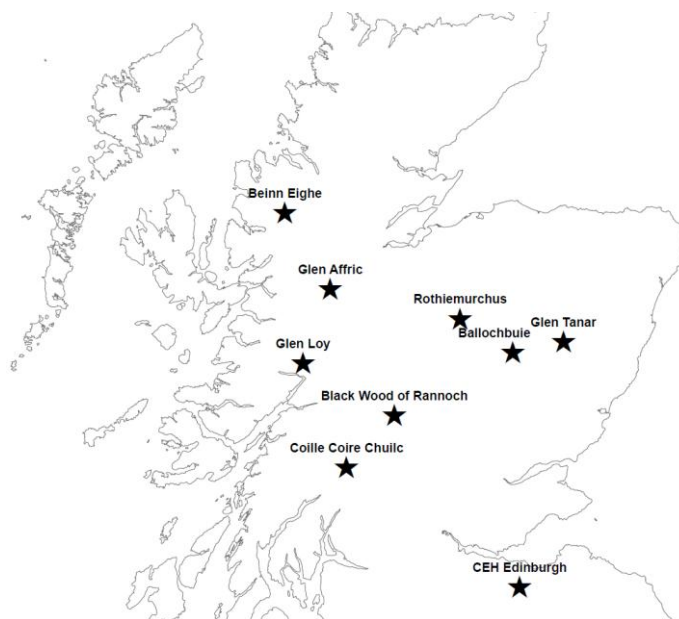
Figure 5.1 Locations of the sampled populations and the study site (CEH Edinburgh).

5.3.2 Experimental setting

Seeds from the 32 mother trees were sampled in winter 2007 and sown on trays (75:25 compost type John Innes 1: sand) in summer 2007. After germination six seedlings per family were transferred to pots of size 0.62 l (diameter 11 cm, depth 9.6 cm) and the trial was divided into six blocks of 32 seedlings each (i.e. one family member per block). The order of families within blocks was randomized. The seedlings were kept under common-garden glasshouse and natural light conditions (glasshouse shaded to avoid excess light) with watering applied 2-3 times per week during the growing season, depending on the weather conditions. Seedlings were potted up in spring 2008 (compost changed to John Innes 3) to pots of size $11 \times 11 \times 12$ cm (length $\times$ width $\times$ depth) and transferred outdoors to a bench 90 cm above the ground in August 2009. The study site at CEH Edinburgh (figure 5.1) is located around 190 meters above sea level and according to the UK Met Office data (Perry and Hollis 2005), has an average GSL of 234 days. The mean temperature is 1.8°C in February and 13.1°C in July. Weather data were regularly recorded at the site and were used to calculate heat sum accumulation in spring (in day degrees, dd). Watering was applied only when wilting was observed due to lack of rain.

Table 5.1 Environmental data for populations and sites. Columns are core pinewood size (CPS; ha) according to Mason, Hampson et al. (2004), growing season length (GSL; days), air and ground frost days per year (FD A, G; days), mean February (MFT; °C) and July temperatures (MJT; °C), family (site) name, altitude (AL; m), aspect (AS), and accumulated temperature (AT, day degrees).

Population	CPS (ha)	GSL (days)	FD A	FD G	MFT (°C)	MJT (°C)	Family	AL (m)	AS	AT (dd)
Ballochbuie (BB)							BB74	500	N	717
	775	108	152	179	-2.0	9.4	BB75	524	NE	687
							BB80	456	NW	772
							BB97	421	N	816
Glen Loy (GL)							GL1868	155	N	1168
	74	187	97	149	0.3	9.7	GL1872	161	NE	1160
							GL1876	197	N	1114
							GL1877	170	N	1149
Glen Affric (GA)							GA1892	205	flat	1073
	1532	204	92	164	0.6	11.5	GA1893	274	NW	990
							GA1897	274	NW	989
							GA1900	270	SE	994
Rothiemurchus (RM)							RM1841	306	SE	951
	1744	216	101	163	1.0	13.0	RM1845	325	flat	928
							RM1846	329	N	923
							RM1848	311	NW	944
Coille Coire Chuilc (CCC)							CCC1801	298	S	1024
	67	223	90	151	1.4	12.2	CCC1806	222	SE	1125
							CCC1807	237	NE	1105
							CCC1809	269	S	1062
Glen Tanar (GT)							GT1851	289	NW	975
	1564	231	84	152	1.8	13.5	GT1856	422	N	810
							GT1858	345	NW	906
							GT1860	330	NW	924
Black Wood of Rannoch (BW)							BW1822	307	N	990
	1011	252	78	162	1.8	13.4	BW1825	257	S	1054
							BW1828	250	N	1063
							BW1830	285	N	1082
Beinn Eighe (BE)							BE21	59	NE	1210
	182	279	61	133	3.4	14.0	BE23	91	NE	1173
							BE26	83	NE	1183
							BE30	17	NE	1259

5.3.3 Chlorophyll fluorescence

Chlorophyll fluorescence was measured in the seedlings on 14 occasions between September 2009 and May 2010. Measurements were taken outdoors between 8.00 am (or dawn) and noon. Two current- or previous-year needles from opposite sides of the main stem and from separate fascicles were used (in some cases needles from branches rather than those from main stem were sampled), and their mean was used as the fluorescence value per plant. Needles were removed from the seedling, and measurements were always taken on the adaxial side. Removal of leaves has been shown to have little effect on the recorded variables (Mohammed, Binder et al. 1995). Due to blocking, measurements for each population and family were spread over the scoring time.

Immediately after removal, needles were dark-adapted for approximately 20 minutes. After dark adaptation, a Handy PEA portable fluorescence measurement system (Hansatech Instruments Ltd, Norfolk, UK) was used to give a saturating light pulse (intensity 2500 μM) resulting in the fluorescence reaching its minimal level (F_0). At this stage all PSII reaction centres are open (Q_A, a primary electron transporter, in an oxidised state). After this, fluorescence quickly rises to its maximal level (F_m), a point where all electron transporters are reduced and reaction centres closed. Variable fluorescence (F_v) is the difference between F_m and F_0, and the maximum quantum yield of primary photochemistry at PSII can be expressed as F_v/F_m (photochemical capacity).

5.3.4 Spring phenology

Initiation of shoot elongation

The height of the seedlings was measured seven times between April 9 and May 31. The main stem was measured in most cases; however, in some seedlings measurements were taken on branches due to damage in the apical meristem caused by snow. In such cases the measurements were always taken on the same branch.

Timing of needle emergence

We scored timing of needle emergence (needle flush) in each seedling twice weekly between May 9 and June 15. We recorded the emergence of new needles around the tip of the buds in the main stem and in the uppermost whorl (max. four branches/buds). An average date was calculated for the branches of a seedling, and using the weather data, we then calculated heat sums on the days of needle flush in the apical bud and branches. The average of these two values was used to describe timing of needle flush in each seedling. In cases where there was no apical bud or if it had been damaged, the mean of the branches was used, and vice versa. Heat sum was calculated as temperature accumulation above +5°C starting on March 13, the first day in 2010 with an average temperature above +5°C that was followed by two more days as warm or warmer.

5.3.5 Statistical analyses

A nested ANOVA (families nested within populations) was used to analyse variation in F_v/F_m and timing of needle flush. Population was considered a fixed factor and families and blocks random. Variation in F_m and F_0 was only examined using means

on each measurement date. A correlation analysis was used to examine relationships between fluorescence parameters and spring phenology. Altitude was chosen as a surrogate for temperature variation as it is a stable measure among years and strongly influences site AT estimates (r=-0.97, p<0.0001). Also, average altitude of families within a population is correlated with average GSL (r=-0.72, p<0.05). A regression analysis was carried out to test for relationships between altitude and population and family means of fluorescence parameters and initiation of growth. Linear regressions were used except in the case of F_v/F_m on March 21 when a polynomial quadratic regression was applied. When used in testing for associations, F_v/F_m was expressed as $1/(1-F_v/F_m)$.

To estimate the date of growth initiation in each family, defined as a 10% increase in mean height, we set the first measurement to 1 and divided the later measurements by the first one to get the percentage increase in height between two dates. For each family, we fitted an exponential curve $y=a+br^X$, where y=mean family height at time X (days since the first measurement), a is a constant term, and b and r are shape parameters estimated for each family. We estimated the number of days since the first measurement (X) required for a 10% increase in mean family height (i.e., when y=1.10), defined the corresponding date and calculated heat sum on that day.

All statistical tests were carried out using GenStat 10.2.0.175.

5.4 Results

5.4.1 Temperature variation at the experimental site

During the study, temperature dropped below 0°C for the first time on November 9, but daily average temperatures stayed above freezing until late November (figure 5.2). The coldest day of the period was January 7 (average temperature -6.2°C). The winter of 2009/2010 was exceptionally cold for Scotland: the long-term (1971-2000)

average winter temperature is 2.7°C, while between December and February 2009/2010 it was estimated by UK Met Office to be 0.24°C. The seedlings were covered in snow from mid-December until January 21.

5.4.2 Photochemical capacity (F_v/F_m)

A distinct seasonal pattern was observed in F_v/F_m, with the peak level reached in October and the minimum in early March (figure 5.2). Between September and December, F_v/F_m means varied between 0.60 on November 27 and 0.80 on October 29, but no statistically significant differences were observed among populations or families within populations on any measurement date (data not shown).

On January 22, significant differentiation among populations was observed (table 5.2). Mean F_v/F_m had dropped to 0.58, and the lowest F_v/F_m was recorded in BE (0.50), and the highest in GT (0.63). On February 5, marginally significant differences were observed among families within populations (data not shown). F_v/F_m kept decreasing until March, reaching its lowest values on March 5, with population means varying between 0.35 in BE and 0.45 in GT. However, the population factor was not significant until March 21 (marginal differentiation, table 5.2), at which point F_v/F_m had started to recover along with site temperatures and varied between 0.55 in BE and 0.64 in GT. A cold spell in late March and early April disrupted the F_v/F_m recovery in April. By the final measurements on May 9, population means had not yet recovered to the level observed before the onset of winter. On April 5, 19, and May 9 differences in F_v/F_m were only observed between blocks (data not shown).

5.4.3 Variation in mean overall F_m and F_0

F_m remained stable between September and mid-November, peaking on October 1, after which it started to drop, reaching its mid-winter minimum on February 19 (figure 5.3). The change in F_m between October 1 and February 19 was -65%. After this, F_m started to recover, dipping again in early April following a late cold period (figure 5.2), but recovering quickly. By May 9, F_m had increased 229% compared to February 19. Smaller changes in F_0 were observed during the study period. Between September 19 and January 22 F_0 decreased 47%, and by May 9 it had reached levels similar to those observed in September.

5.4.4 Associations with environmental variables

On January 22, families from higher altitudes showed higher F_v/F_m (for altitude: β_0=0.970, β_1= 0.0015, p<0.001, R^2=37%, figure 5.4). Among populations, average altitude explained 74% of the variation in F_v/F_m (β_0=0.938, β_1=0.00160, p<0.01).

A moderate correlation was observed between family means of F_v/F_m on January 22 and March 21: generally, seedlings with higher F_v/F_m in January also had higher values in March (r=0.56, p<0.001). However, on March 21, environmental trends were not linear. Lowest means were recorded in BE (0.55) and BB (0.58), while in other populations means were 0.60 or higher. The relationship between family means of F_v/F_m on March 21 and altitude was significant and explained 18% of the variation (β_0=1.110, β_1=0.0034, β^2=-0.0000056, p=0.022, figure 5.5). Among populations, average altitude explained 65% of the variation in mean F_v/F_m (β_0=0.998, β_1=0.00433, β^2=-0.00000740, p<0.05).
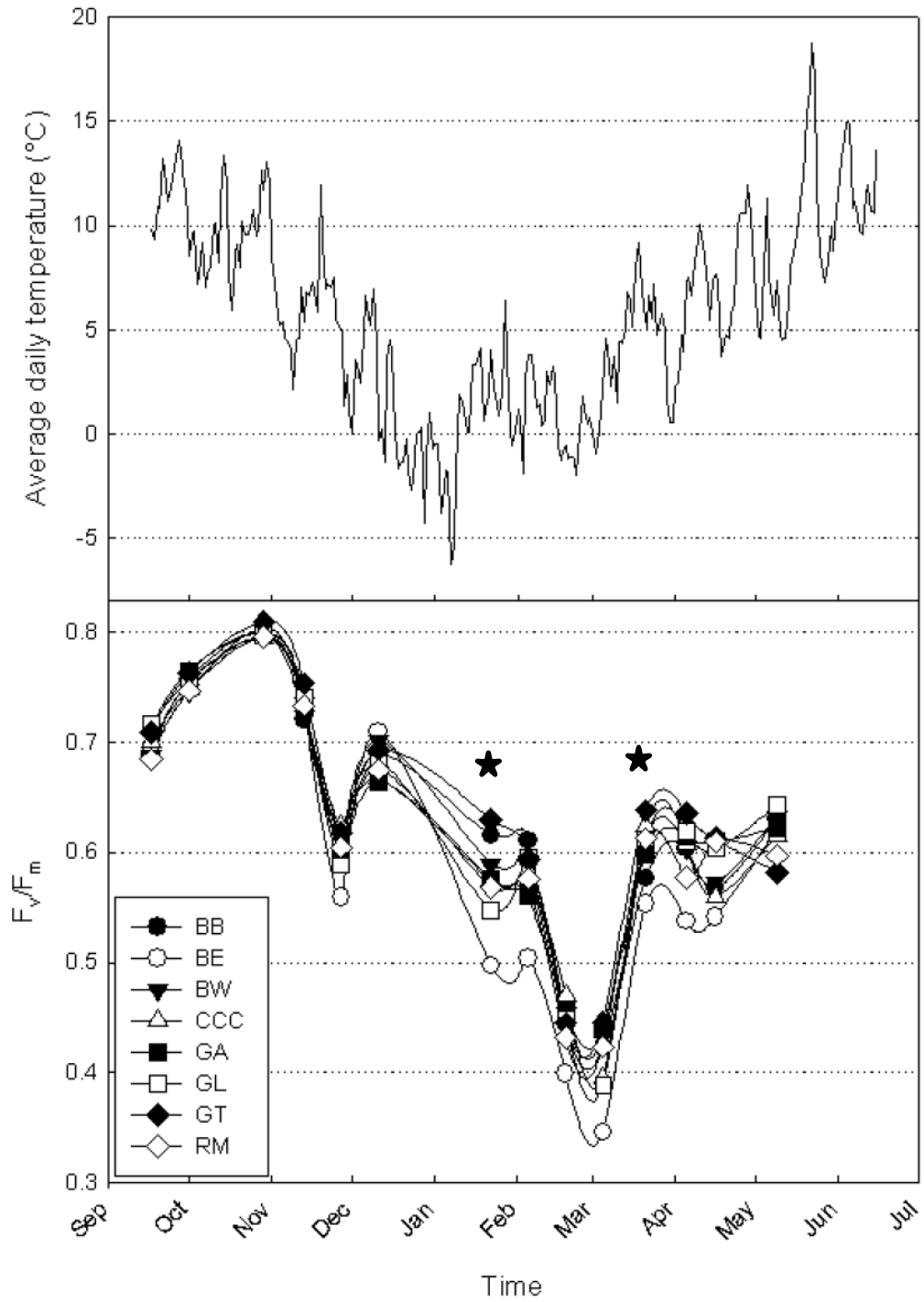
Figure 5.2 Average daily temperatures at the experimental site between September 17 2009 and June 15 2010 and variation in population means of F_v/F_m between September 17 2009 and May 9 2010. The dates on which significant differences among populations were found are marked with star symbols.
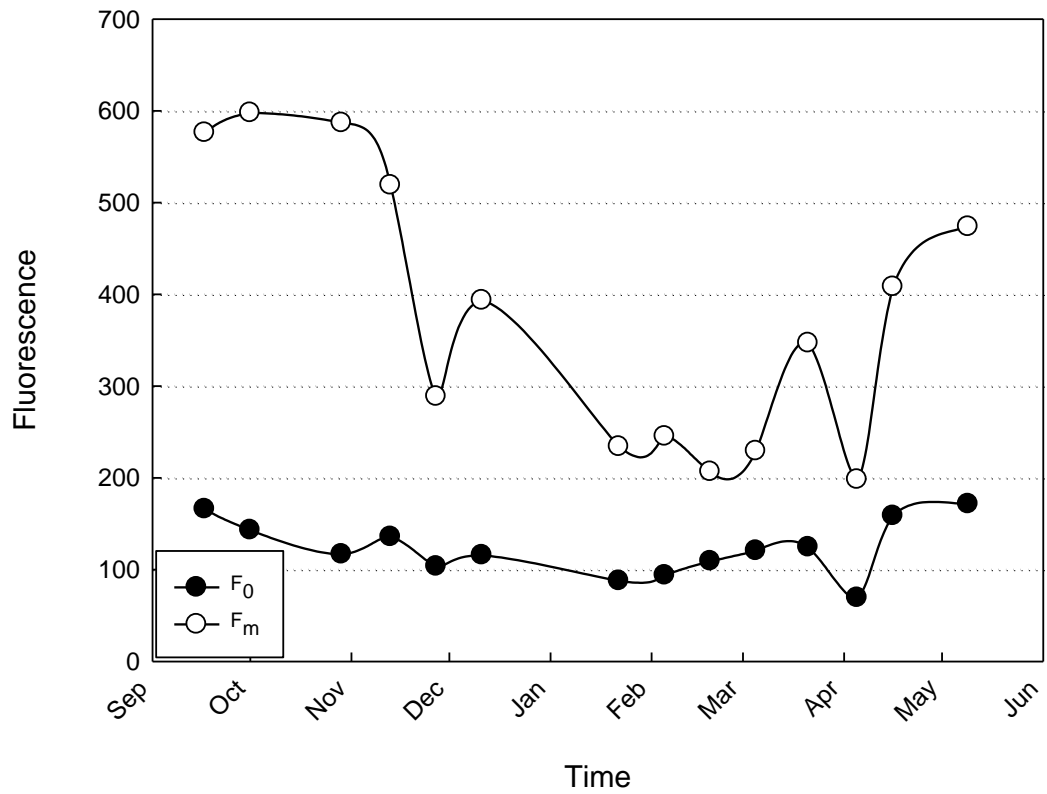
Figure 5.3 Variation in overall means of F_0 and F_m between September 17 2009 and May 9 2010.

Table 5.2 ANOVA tables and mean squares of factors for F_v/F_m on January 22 and March 21 and timing of needle flush. ns=not significant ($p>0.05$), *=$p<0.05$, **=$p<0.01$.

		Trait		
Factors:	df	F_v/F_m - Jan 22	F_v/F_m - March 21	Needle flush (dd)
Population	7	0.0398 **	0.018 *	3814 ns
Families within population	24	0.00949 ns	0.00729 ns	1976 *
Blocks	5	0.00804 ns	0.0298 **	1349 ns
Residual	155	0.0162	0.00774	1105

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5.4.5 Spring phenology

Initiation of shoot elongation

Exponential curves fitted to describe the early phase of height growth among families explained 91.4-99.8% of the variation ($p<0.01$ in all families). Shoot elongation in families was initiated between late April (family RM1846 at 57 dd) and late May (family BE21 at 181 dd). Among populations, mean heat sum requirement was lowest in RM (mean 87 dd) and highest in BE (mean 141 dd).

Needle flush

Needle flush was initiated later than shoot elongation and occurred between mid-May and early June. Among families, the lowest mean heat sum requirement was observed in BB75 (163 dd) and the highest in BW1830 (247 dd). Population means were lowest in BB (181 dd) and highest in BE (216 dd). However, significant differentiation in needle flush was observed only among families within populations (table 5.2). A strong correlation was observed between timing of needle flush in the apical bud and in the branches ($r=0.85$, $p<0.001$). A positive correlation was also found between the family means of initiation of shoot elongation and needle flush ($r=0.63$, $p<0.001$).

Associations between spring phenology, photochemical capacity, and environment

Among families, photochemical capacity at cold temperature (F_v/F_m on January 22) was significantly negatively correlated with needle flush ($r=-0.50$, $p<0.01$) but not with initiation of shoot elongation ($r=-0.27$, $p=0.14$). The correlation among

population means of F_v/F_m and needle flush was similar in direction (r=-0.46) but not significant (p=0.25).

Among families, altitude was significantly associated with both timing of initiation of shoot elongation (β_0=130.06, β_1=-0.083, p<0.05, R^2=16%) and needle flush (β_0=214.72, β_1=-0.067, p<0.05, R^2=13%, figure 5.6). Among populations, average altitude explained 40% of the variation in needle flush (β_0=216.17, β_1=-0.072, p=0.056); a regression with initiation of shoot elongation was not statistically significant (β_0=129.44, β_1=-0.081, p=0.09, R^2=30%).
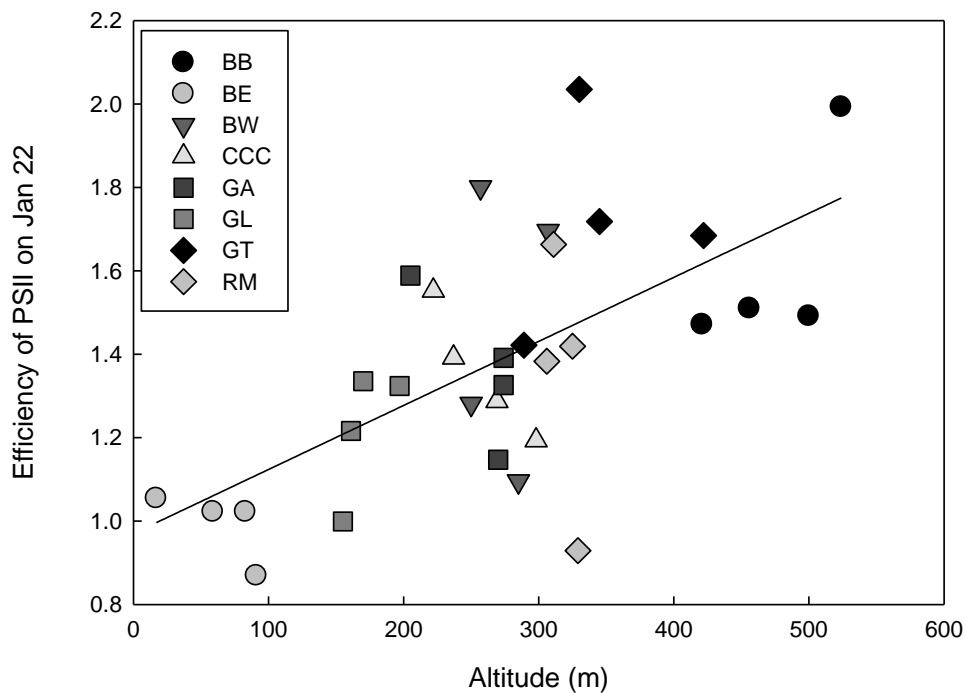
Figure 5.4 Relationship between altitude at home location and family means of efficiency of PSII ($F_v/F_m/(1-F_v/F_m)$) on January 22 (β_0=0.970, β_1=0.0015, p<0.001, R^2=37%).
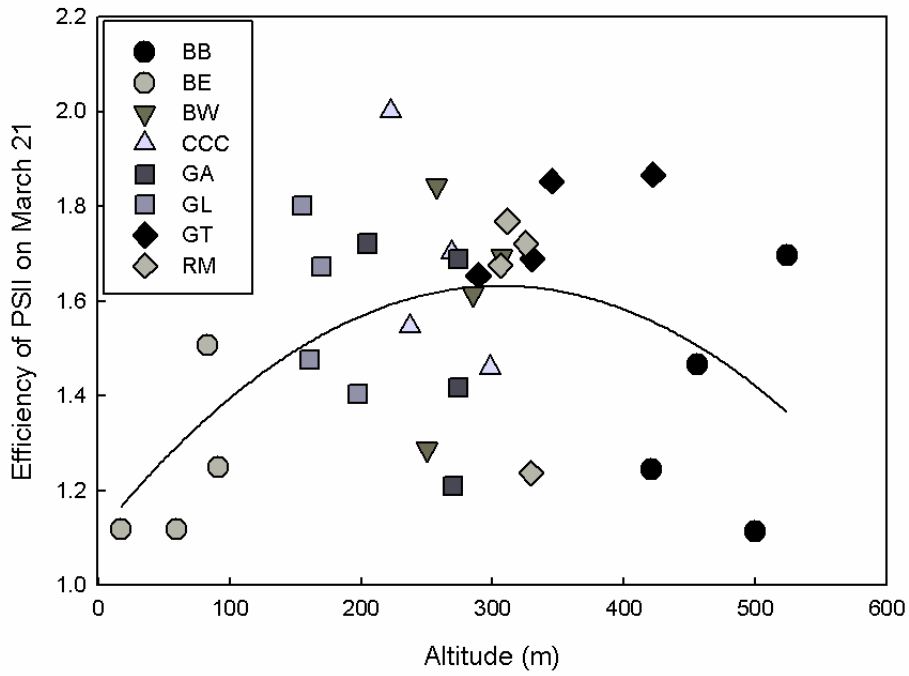
**Figure 5.5** Relationship between altitude at home location and family means of efficiency of PSII ($F_v/F_m/(1-F_v/F_m)$) on March 21 (β_0=1.110, β_1 =0.00341, β^2=-0.0000056, p=0.022, R^2=18%).
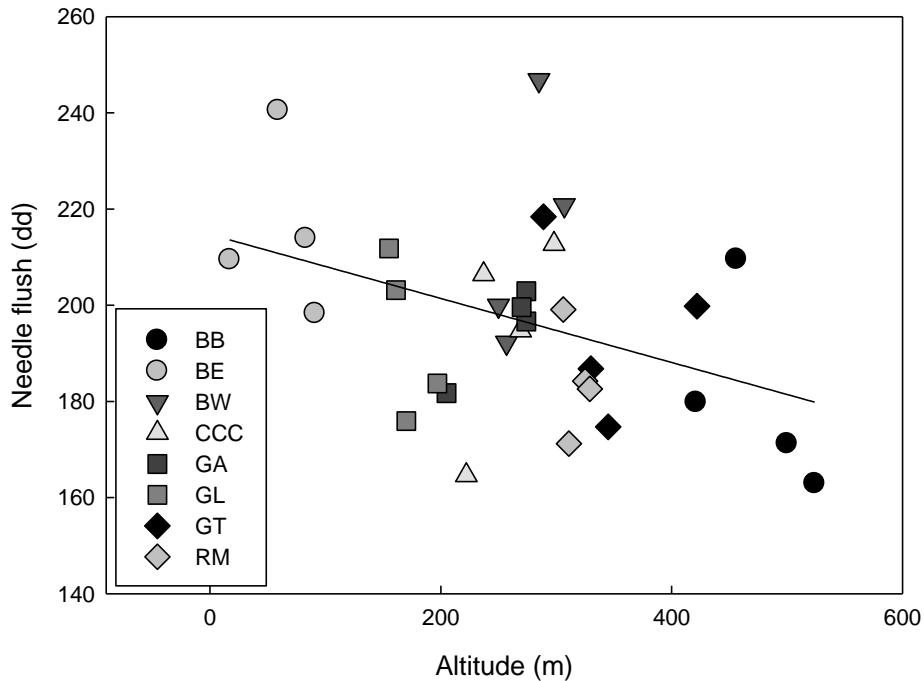

Figure 5.6 Relationship between altitude at home location and family means of needle flush (β_0=214.72, β_1=-0.067, p<0.05, R^2=13%).

5.6 Discussion

In this study, we examined variation in seasonal patterns of photochemical capacity in Scots pine seedlings from eight climatically diverse Scottish populations under natural climatic conditions. F_v/F_m showed a distinct seasonal trend, remaining at relatively high levels until December and decreasing towards mid-winter following a period of over two weeks with temperatures below or close to 0°C. Populations responded differently to the change in temperature, with the largest drop in photosynthetic capacity being observed in seedlings from the climatically mildest conditions. F_v/F_m started to recover in March, and by early May, it had reached the same level observed in early December. Evidence of genetic differentiation was also found in spring phenology. Initiation of shoot elongation and needle flush were earlier in families and populations from cooler areas.

5.6.1 Seasonal variation in photochemical capacity

Similarly to what has been observed in Scots pine growing in northern Sweden (Ottander, Campbell et al. 1995), F_v/F_m showed a seasonal pattern with least activity being recorded in mid-winter. However, photochemical capacity in autumn and early winter remained at a higher level in Scotland: in December, mean F_v/F_m was still 0.7 after which it decreased in response to a severe cold period. In northern Sweden, F_v/F_m in Scots pine can start to decline in late August and reach ~0.20 by mid-December (Lindgren and Hällgren 1993; Ottander, Campbell et al. 1995), and in a *Quercus suber* L. trial in Spain, the decline towards minimum values in early February started in November (Aranda, Castro et al. 2005). Seasonal variation in photosynthetic activity is caused by cool temperatures either limiting or inhibiting CO_2 assimilation while light absorption continues at PSII, resulting in an overexcitation of the photosynthetic machinery, photooxidative damage and eventually photoinhibition (Öquist and Huner 2003). The differences among studies carried out at different locations can be explained by variation in temperature conditions during autumn and winter (e.g. Öquist and Huner 2003). During our

study, freezing temperatures did not occur until early November, and daily average temperature stayed above 0°C until early December. This indicates that in mild climates, Scots pine could remain photosynthetically active well into the late autumn or early winter. In Sitka spruce (*Picea sitchensis* Bong. (Carr.)) seedlings of Alaskan origin growing in southern Scotland, dry mass can double between autumn and spring (Bradbury and Malcolm 1978). However, it is possible that beside CO_2 fixation, electrons from PSII are also used for other processes such as photorespiration or nitrogen metabolism (e.g. Maxwell and Johnson 2000). Patterns of photosynthetic activity among Scots pine populations growing in their native environments may vary considerably due to substantial variation in winter temperatures.

Following the mid-winter dip, F_v/F_m started to recover in March, along with average daily temperatures. However, the sensitivity of F_v/F_m to temperature was seen in April when its rise was halted after a cold period in late March. Such immediate responses indicate that among-year variation in these patterns could be significant depending on temperature conditions. By early May, F_v/F_m had recovered to levels similar to those observed in December. A similar trend was found in Swedish Scots pine by Ottander, Campbell et al. (1995) who reported a steady increase in F_v/F_m starting in March towards maximum summer values in late May; in *Q. suber* in Spain, the recovery started somewhat earlier (Aranda, Castro et al. 2005). While the recovery can take several months under natural conditions, in the laboratory at 20°C, F_v/F_m in Scots pine increased from 0.1 to 0.8 in three days (Lundmark, Hedén et al. 1988).

A reduction in F_v/F_m was caused by decreases in both F_m and F_0, although changes in F_m were somewhat bigger. Similar patterns in pine have been reported before (Strand and Lundmark 1987; Lindgren and Hällgren 1993; Ottander, Campbell et al. 1995). These patterns suggest excess energy absorbed by PSII is being released through non-photochemical dissipation. A decrease in F_v/F_m has been shown to be correlated with loss of chlorophyll and proteins associated with light-harvesting antennae and PSII, while an increase was observed in the amount of xanthophyll (Ottander,

Campbell et al. 1995), a carotenoid that protects chlorophyll from photooxidation, and in non-photochemical quenching capacity. How these biochemical processes vary among Scots pine populations that differ in the timing of cold hardiness is not yet known. In spring, opposite changes occur as F_v/F_m increases (Ottander, Campbell et al. 1995). In our study, the recovery of F_m in spring was slower than that of F_0: by early May F_0 had reached the level observed in September, while F_m had reached only 79% of its maximal value recorded in early October.

5.6.2 Variation among populations

Cold winter temperatures resulted in a decline and significant differences in F_v/F_m among populations on January 22. The response was largest in BE, a low-altitude population located in maritime north-western Scotland, while populations from cooler, high-altitude locations in eastern Highlands showed higher F_v/F_m. The immediate response to cold in December is unknown as snow coverage prevented measurements until late January. Differences in photochemical capacity at low temperature could be due to variation in the development or level of cold hardiness. Throughout the winter, BE had lowest F_v/F_m, although populations were not significantly different until March 21. Mean annual temperature varies greatly among native pinewoods, resulting in variation in temperature-dependent variables such as growing season length which potentially acts as a strong selective force. Our results show evidence of environment-driven genetic differentiation among Scottish populations and are in accordance with other studies on trees showing adaptive differentiation in response to cold temperatures (reviewed in Howe, Aitken et al. 2003). However, the geographical scale in our study is much smaller than in previous common-garden studies on Scots pine, suggesting that in environments with high spatial heterogeneity such as Scotland, genetic differentiation could occur at short distances. Autumn cold hardiness in young Fennoscandian Scots pine seedlings has been shown to be genetically inherited (Nilsson and Walfridsson 1990) although maternal effects can have some temporary impact on the trait (Dormling and Johnsen 1992; but see Ruotsalainen, Nikkanen et al. 1995). Within populations, cold

hardiness has been found to develop similarly between different age classes (Beuker, Valtonen et al. 1998). It is possible that the trait under selection is not level of cold hardiness *per se*, but its timing (Repo, Zhang et al. 2000). Interestingly, no mortality occurred in our study material despite severe cold. This could be due to the fact that three-year old seedlings have already passed their most sensitive developmental stage (Persson and Ståhl 1990).

We have demonstrated seasonal variation in photochemical capacity among multiple populations of an evergreen conifer growing under natural climate conditions. This approach has previously been successfully applied to Spanish populations of *Q. suber* in which patterns of variation among populations were very similar to ours (Aranda, Castro et al. 2005). Following growth cessation, cold hardiness develops in response to shortening days and cooling temperatures (Gillies and Vidaver 1990), and it has been shown in Scots pine that hardiness develops in parallel with declining rates of photosynthesis (Repo, Leinonen et al. 2006). F_v/F_m has been shown to be an informative stress indicator in a range of species (Mohammed, Binder et al. 1995), and for instance Lindgren and Hällgren (1993) used chlorophyll fluorescence to demonstrate that northern Swedish Scots pine populations initiate hardening and are hardier than southern populations when growing in the same environment. In this study we did not determine possible differences in timing of cold hardiness, but hardening in Scotland could be delayed compared to boreal conditions due to a milder climate.

The marginally significant population differentiation observed on March 21 suggests population differences in timing of dormancy release. The lowest mean was recorded in BE, and in other populations, with the exception of BB, mean F_v/F_m had already reached 0.60. The slow response of seedlings from BB, the population from the coldest location included in the study, is surprising as its response was similar to that of the seedlings from maritime BE. Other studies have found contrasting de-hardening patterns both among and within species. De-hardening has been shown to occur simultaneously with increasing photosynthesis in Scots pine (Repo, Leinonen et al. 2006), but no differences in timing were found among northern and southern

Finnish populations (Beuker, Valtonen et al. 1998). A Swedish Scots pine population was shown to lose root cold hardiness later than four Scottish populations (Perks and McKay 1997), while in mountain birch (*Betula pubescens ssp. czerepanovii* (Orl.) Hämet-Ahti), the slowest de-hardening was observed in an oceanic population (Taulavuori, Taulavuori et al. 2004). These findings suggest that different coping mechanisms have evolved in different places. Climate data suggest that although average monthly temperatures start rising after February in all populations, only in BB does the average number of both air and ground frost days remain very similar between January and March, possibly creating a local selective force that has resulted in delayed response to rising temperatures. However, at BB, the estimates of air and ground frost days were not recorded in the exact area of the pinewood, but were based on the partially interpolated UK Met Office data (Perry and Hollis 2005). Due to the temperature sensitivity of F_v/F_m, a more intensive sampling scheme in early spring at the time when air temperature starts to rise would be required to look at this scenario in more detail. Also, photosynthetic activity measurements could be taken on trees growing in their home environments, along with regular temperature recordings, and these patterns could be compared between pinewoods from different parts of Scotland.

5.6.3 Spring phenology

Evidence of adaptive differentiation among populations was found in spring phenology as well. Shoot elongation started earlier than needle flush, but only needle flush was significantly correlated with photochemical capacity under cold temperatures (F_v/F_m in January). Bud flush occurs in spring after the genetically-determined chilling and heat sum requirements have been met (Dougherty, Whitehead et al. 1994; Aitken and Hannerz 2001). Perks and Ennos (1999) observed significant differentiation in timing of needle flush among four native pinewoods in Scotland, but the variation could not be linked to environmental factors. Applying the commonly-used criterion of above +5°C average daily temperatures to define the start of heat sum accumulation, we found significant differences only among families

within populations. However, it is well-known that environmental cues for growth initiation vary among populations of various pine species (e.g. Dougherty, Whitehead et al. 1994). In Scots pine, a Scottish population was found to have a longer chilling requirement compared to Northern European populations, indicating a mechanism preventing initiation of growth under mild winter conditions (Leinonen 1996). Similar variation might also be found within Scotland due to extensive variation in annual temperature features among populations. However, in addition to having a significant genetic component, quantitative characters are often strongly influenced by the environment (Falconer and Mackay 1996), and a trait's expression might differ if environmental conditions change. In the study by Leinonen (1996), an extended chilling treatment decreased population differences in the sum requirement, suggesting that patterns of variation in timing of growth initiation could vary temporally if trees are exposed to varying winter and spring temperatures in different years. For example, Beuker (1994) reported temporal variation in the required heat sum among North European Scots pine populations growing at two sites in southern and south-eastern Finland, and also growth cessation in first-year seedlings has been shown to be influenced by photoperiod and temperature (Koski and Sievänen 1985). The contribution of temporal climate variation could be determined by observing variation in the same samples in different years. In Scotland, variation in winter severity from exceptionally warm to severely cold has been noted for instance by Harrison (1997), and in an outdoor common-garden trial located close to our study site, the stage of budburst and leaf elongation in seedlings from Scottish populations of *Betula pubescens* Ehrh. and *B. pendula* Roth. were more advanced in late April 1984 than at the same time in 1986 (Billington and Pelham 1991).

The significant associations between family means and altitude found in our study suggest that timing of growth initiation is of adaptive importance. Growth started earlier in populations from cooler areas, a pattern similar to that found among 14-year old Scots pine saplings from 108 Eurasian populations (Steiner 1979) and between about 60-year old trees from northern and southern Finnish populations (Beuker 1994). Although annual heat sum requirements among populations varied in the study by Beuker (1994), the ranking of the populations was similar in different

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years. Considering the effects of extended chilling on population-specific heat sum requirements for bud flush (see above, Leinonen 1996), it is possible that the strength of these associations varies temporally. The significant variation observed among families within populations suggests that timing of needle flush is genetically inherited and that adaptive variation is maintained within populations, possibly as a result of gene flow among sites with differing selective pressures (Howe, Aitken et al. 2003).

In summary, we have demonstrated seasonal variation in photosynthetic activity in Scots pine in Scotland, and found evidence of genetic differentiation in photochemical capacity at low winter temperatures among eight pinewoods. Patterns of variation in F_v/F_m were found to differ from those in boreal populations, possibly due to the milder climate of Scotland. Winter temperatures affected populations differently: photochemical capacity in populations from high-altitude locations was least sensitive to cold. Patterns of spring phenology mirrored those for photochemical capacity: growth was initiated earlier in trees from cooler locations. The traits studied are partly influenced by temperature, thus there might be significant among-year variation in these patterns. Our results have demonstrated genetic differentiation among native pinewoods and suggest that populations may be locally adapted at a fine spatial scale. To prove local adaptation reciprocal transplant experiments would be required to examine the effects of transfers between different environments and to test whether populations have highest fitness at their home site (Kawecki and Ebert 2004). Nonetheless, our findings indicate that patterns of environmental and genetic variation among native pinewoods should be taken into account when defining seed transfer guidelines for Scots pine in Scotland. Variation in other climate-related traits should also be assessed as different aspects of climate may vary among pinewoods.

5.7 Acknowledgments

6. Conclusions

This study examined how environment has shaped genetic differentiation in quantitative traits of Scots pine in Scotland. Environment-driven genetic differentiation is characteristic of many forest trees that have large geographic ranges covering highly heterogeneous environments (Howe, Aitken et al. 2003; Savolainen, Pyhäjärvi et al. 2007; Aitken, Yeaman et al. 2008), but we have only limited understanding regarding the adaptive evolution of Scots pine in Scotland. Some provenance trials were initiated by the Forestry Commission starting in the 1920s, but the experimental designs allowed comparisons only between Scotland as a whole and mainland European populations and showed that continental populations do not perform as well as native populations in Scotland (Lines and Mitchell 1965; Worrell 1992). Scottish populations have been included as representatives of maritime sites in early range-wide studies of variation across Eurasia (Wright and Bull 1963; Read 1971; Karrfalt, Gerhold et al. 1975; Steiner 1979), and while the sampled Scottish pinewoods might occupy more maritime environments than those on the continent, the possibility of variation within Scotland was not considered in these experiments. Today, 84 native pinewood sites are recognized by the Forestry Commission, the total core area of which adds up to approximately 18,000 ha (Mason, Hampson et al. 2004). Although geographic distances among pinewoods in Scotland may be relatively short, the possibility remains that there may be genetic differentiation across this area if environmental gradients are sufficient to result in abrupt changes in selection pressure. Due to variation in the distance from the Atlantic coast and to topography, climate conditions among pinewood sites can be expected to vary, and some evidence of adaptive differences among a small number of populations have already been documented (Perks and McKay 1997; Perks and Ennos 1999). Variation in growth and phenology among populations from different parts of Scotland has also been reported in *Betula pendula* (Worrell, Cundall et al. 2000).

Other studies on adaptive variation in Scots pine have revealed population differences in a number of quantitative traits such as timing of bud flush (Steiner 1979), growth and timing of bud set (Wright and Bull 1963), and onset of cold

hardiness (Hurme, Repo et al. 1997) among continental European and Asian populations. However, it should be borne in mind that genetic differences do not directly prove local adaptation which is defined as a population having its highest fitness at its home site (Kawecki and Ebert 2004). Evidence for local adaptation can only be derived from reciprocal transplant experiments in which the performance of transferred populations can be compared to that of the local population at its home site. In countries where Scots pine is a commercially important tree species, extensive provenance experiments are already decades old (e.g. Eiche 1966; Shutyaev and Giertych 1998) and continue to be used for scientific research (Persson and Ståhl 1990; Rehfeldt, Tchebakova et al. 2002; Savolainen, Pyhäjärvi et al. 2007). In such provenance trials in the northernmost parts of Sweden, local populations often demonstrate higher survival than those from other regions (Eiche and Andersson 1974). On the basis of results of such trials, Persson and Ståhl (1990) concluded that survival of a population is decreased by 7% when transferred 1° higher in latitude and by 16% per 100 m-increase in altitude. Transfers further south to milder environments result in an increase in survival. These studies indicate that genetic differentiation in response to local climate has occurred in many parts of the species' range.

6.1 Climate variation in Scotland

The long-term UK Met Office data (Perry and Hollis 2005) was used to explore spatial climate variation among the 21 native pinewood sites. The results revealed considerable differences in temperature and rainfall features among the pinewoods, and generally sites located in the west close to the coast had longer growing seasons, milder winters, and more annual rainfall were located in the west close to the coast, while those further east were found to experience shorter growing seasons, colder winters, and less rainfall. Previously, the seed zones designated by the Forestry Commission for the control of the germplasm movement among the 84 native pinewoods were found not fully to match patterns of environmental variation (Salmela, Cavers et al. 2010). If the heterogeneous environment has given rise to

genetically differentiated populations, it is possible that current guidelines promote planting with stock that will have a reduced likelihood of survival due to differences between home and away site conditions.

6.2 Adaptive differences among Scots pine populations

6.2.1 Spring phenology

At the beginning of the second growing season, evidence of genetic differentiation among 21 populations was found in timing of bud flush which was generally earlier in seedlings from sites with shorter growing seasons and colder winters. In the following year, no such trends were discovered. However, a similar trend was observed at the beginning of the fourth growing season in seedlings from eight populations and 32 families growing outdoors. These patterns indicate that the timing of growth in different populations is optimized according to the home site conditions: when the growing season is of limited length, for instance at high-altitude sites, a lower temperature sum is required for growth initiation compared to warmer sites (Steiner 1979). However, in mild maritime climates, starting too early in spring might expose new growth to damaging late frosts. It is possible that environmental conditions in the glasshouse during the winter and spring before bud flush in 2009 differed from those in 2008 which might explain the differences in the trait's expression.

6.2.2 Response to droughting and winter/spring temperatures

Seedlings were tested for response to drought stress in their third year. Chlorophyll fluorescence was used to examine how seedlings from 20 families in five populations from areas with contrasting levels of annual rainfall varied in response to droughting.

The results were surprising as the response of populations was not related to rainfall; the largest reduction in photochemical capacity after two weeks of droughting was observed in seedlings from both the driest and the wettest population (in terms of annual rainfall). However, family means of photochemical capacity were associated with the altitude at their home site and possibly also with moisture deficit which estimates site dryness during the summer and can be obtained using the Ecological Site Classification (Pyatt, Ray et al. 2001). These patterns suggest that rainfall alone might not be a good descriptor of site moisture conditions in spatially complex and heterogeneous environments such as Scotland, but because moisture deficit estimation is partly based on interpolation, the accuracy of these estimates is uncertain, and it is not known whether variation in this factor is really responsible for the observed phenotypic differences. Also, the populations for this study were chosen according to the precipitation data, and in retrospect, sampling based on a gradient in moisture deficit might have been a better strategy.

Chlorophyll fluorescence was also used to study how seasonal photochemical capacity varied in seedlings from 32 families in eight populations from different temperature regimes in response to changing outdoor temperatures between autumn and spring. During the study period, some of the seedlings were exposed to unusually cold winter temperatures, and although the immediate response to decreasing temperature could not be measured, significant population differences persisted after a few weeks' exposure to cold in January. The response to cold was associated with the home environment of the seedlings: those from cold-winter sites at high altitudes showed the smallest decline in photochemical capacity, suggesting that populations might have differed in cold hardiness at the time of the measurement. Population differences were also observed in recovery of photochemical capacity in spring: the response to rising temperature in late March was smallest in the populations from the warmest and coldest sites. The slow recovery of the maritime population might be related to avoidance of premature initiation of growth in spring, while it could be that in the population from the coldest site, the high number of air and ground frost days despite increasing spring temperatures has resulted in a delayed response compared to sites where increasing monthly mean temperature is associated with a decreasing

number of frost days. The results from the outdoor experiment also suggest that Scots pine in Scotland could remain photosynthetically active further into the autumn compared to Scandinavian pines growing at their home sites. However, additional measurements beside chlorophyll fluorescence would be required to explore photosynthesis in more detail, and more frequent measurements (e.g. once a week) would allow examining finer-scale variation in these traits.

6.2.3 Effects of the environment on quantitative trait expression

Quantitative traits are often under a complex polygenic and environmental control (Falconer and Mackay 1996). For instance, both growth initiation and cessation are known to be genetically inherited in many forest trees which is seen as phenotypic differences among populations and families in common-garden studies (Howe, Aitken et al. 2003). However, bud flush is also controlled by chilling and heat sum requirements, (Aitken and Hannerz 2001), while growth cessation is influenced by photoperiod and temperature accumulation (Koski and Sievänen 1985). As a result, the same trait can be expressed differently in contrasting environments (e.g. Campbell and Sorensen 1978, Falkenhagen 1979). It is also possible that the environment sometimes 'disguises' the genetic component in these traits: in Scots pine, a prolonged exposure to chilling has been found to decrease differences in heat sum requirement among Scots pine populations (Leinonen 1996), and among populations samples across a latitudinal gradient of 20°, timing of bud set varied most in the coolest treatment, while in very warm conditions no such differences were observed (Oleksyn, Tjoelker et al. 1998). Of interest would be to determine how patterns of quantitative trait variation vary temporally in the same set of samples. Beuker (1994) found that heat sum requirements among Northern Finnish Scots pine populations growing in southern Finland varied temporally, although the ranking of populations remained similar each year. In a trial of Scottish birch populations growing near Edinburgh, bud flush was much more advanced in April 1984 than at the same time in 1986 (Billington and Pelham 1991). Climate conditions can vary temporally, and the differences among years can results from

interactions of environmental and genetic factors. Considering the significant temporal fluctuation in temperature conditions in Scotland (e.g. Harrison 1997), similar among-year differences in phenology could also be expected in the Scots pine seedlings growing outdoors.

6.2.4 What is local adaptation in temporally unstable environments?

Many quantitative traits in natural populations are known to be under selection which is expected to erode genetic variation (Falconer and Mackay 1996). Yet genetic variation has been found in a wide range of traits from fitness to morphological characters, and in fact, traits closely related to fitness often have higher levels of genetic variation than those under weaker selection (Houle 1992; Merilä and Sheldon 1999). The question of how adaptive genetic variation is maintained has been addressed by numerous evolutionary biologists in the last few decades (Falconer and Mackay 1996; Roff 1997 and references therein), but it seems that there is no definite answer and very few empirical studies have been carried out. In forest trees, spatially varying selection and high levels of gene flow among populations from heterogeneous environments have been considered major factors in explaining why adaptive traits show significant within-population variation in common-garden experiments (Howe, Aitken et al. 2003; Yeaman and Jarvis 2006; Savolainen, Pyhäjärvi et al. 2007). Theory predicts that fluctuating environment and selection can help maintain variation in genetically-determined traits if generations overlap and if some groups are immune to selection (Ellner and Hairston 1994), and such processes could affect long-lived trees with decreasing mortality towards later life stages (Howe, Aitken et al. 2003; Westfall and Millar 2004).

This study found that in addition to extensive spatial climate variation, the climate in Scotland also has a strong temporal component, and temperature variables can show extensive among-year fluctuation. Climate data from the period 1960-2000 suggest that the extent of temporal variation varies within Scotland, and that the most

maritime sites on the west coast are characterised by more stable environments. Further east, at more continental sites, temporal variability increases. Vasseur and Yodzis (2004) have reported similar trends in a large number of time series of climate variables from different parts of the world. Interestingly, three high-altitude populations in areas with the highest level of temporal climate variation were also characterised by high levels of variation in timing of bud flush at the beginning of the second growing season. The cause of this variation could not be determined, but it could results from the seedlings being exposed to a novel environment (glasshouse) which in turn results in release of 'cryptic genetic variation' (Hoffmann and Merilä 1999; McGuigan and Sgrò 2009). This variation might not be expressed in the natural habitats of the populations. Indeed, Conner, Franks et al. (2003) found that in wild radish, a larger proportion of the phenotypic variation was due to genetic factors in the glasshouse than in the field.

However, other explanations can also be examined. In addition to gene flow and spatial within-population heterogeneity, temporally varying climate could be involved in shaping patterns of adaptive variation in Scots pine in Scotland. Most studies on adaptive genetic differentiation among populations have focused on associations between population means and averages of different climate variables, and the possibility of adaptations to fluctuating variables has not received much attention. However, natural environments are not stable (e.g. Vasseur and Yodzis 2004), and it is possible that the role of fluctuating selection in nature is bigger than previously thought (Bell 2010). This factor could also contribute to range limits in different species if temporal instability of the environment increases towards range peripheries, but so far the potential role of genetic variation in limiting distributions has received more attention (e.g. Eckert, Samis et al. 2008). Some studies on Scots pine have suggested that peripheral Eurasian populations are not optimally adapted to their home sites, possibly due to gene flow from range centres (Rehfeldt, Tchebakova et al. 2002; Savolainen, Pyhäjärvi et al. 2007), but on the other hand, these studies have sampled geographic areas large enough to contain populations exposed to variable levels of temporal fluctuations so that determining the conditions to which populations have adapted to in the past might be difficult, especially in the

most variable environments. It is unlikely that adaptation in such environments occurs in response to climate averages because in nature, populations are never exposed to them. However, when the environment fluctuates around a long-term average, patterns of adaptive variation in populations could resemble adaptation to averages if the within-population sampling is extensive enough. In a species like Scots pine, adaptation in seedlings could occur in response to the environmental conditions during their early development. However, older saplings and trees might have experienced different conditions and selection pressure which could be reflected in genetic differences among age classes. One population could in fact consist of different 'adaptation cohorts' and that the number of such groups could be associated with the extent of temporal variation in selection. The response of different species to environmental fluctuation is likely to vary depending on their life cycles and life history characteristics. For instance, if all individuals in a population are equally affected by changing selection pressure, temporal variation could result in a change in population means but not in the amount of genetic variation.

If temporal climate variation is affecting patterns of adaptive variation in Scots pine in Scotland, similar observations could be expected in other traits and species, too. Availability of long-term climate data and samples from spatially and temporally varying sites will allow further examinations on this topic. In addition to phenotypic assessments, it would be interesting to examine whether the extent of temporal variation in environment is associated with the level of genetic substructuring within populations. However, such studies would also benefit from more detailed investigations on basic pinewood biology, many aspects of which are currently unknown in Scotland.

6.3 Future research recommendations

In this study, mainly phenological variation among Scottish pine populations was assessed. In the northern hemisphere, differences in phenology are expected to be associated with temperature variation which was shown to be considerable within

Scotland. Following these studies on spring phenology, it would be interesting to examine whether populations differ also in their timing of growth cessation in autumn. Such differences have been reported along a latitudinal cline in northern Europe (Repo, Zhang et al. 2000). Based on the photochemical capacity data from the outdoor trial, differences in timing of cold hardiness in autumn could be expected.

To examine how environmental variation affects the expression of adaptive traits, it would be beneficial to continue monitoring the same set of outdoor seedlings in different years. The winter of 2009/2010 was the coldest in Scotland in ~40 years, and the patterns of photochemical capacity and growth initiation might differ in milder (or even colder) conditions. However, temperature is not the only factor that varies within Scotland, and in order to better understand pine's adaptation to local climates, variation in other traits should also be examined. Due to a highly heterogeneous environment within a small geographic area, adaptation in Scots pine could occur at a very fine scale, as in Douglas-fir in the Pacific Northwest (e.g. Campbell 1979), and spatial patterns of adaptation might be complex if factors driving adaptation vary across different geographic gradients (i.e., latitude, longitude, or altitude). As this study was carried out in common-garden conditions, it cannot be estimated how transfers across environmental gradients affect different populations. For such studies, long-term trials with extensive sampling need to be established and regularly maintained across Scotland in contrasting environmental conditions and carefully looked after. Studies on the evolution of native pinewoods would also benefit from finer-scale climate data, as the current Met Office data are available in 25 km^2 squares which might have very variable levels of within-grid variation in topographically complex areas.

Genetic studies on native pinewoods have generally sampled multiple populations from different parts of Scotland, but more ecological studies are often limited to fewer sites. More interdisciplinary research is needed in improving the understanding of the biology of native pinewoods as molecular marker and quantitative trait studies can only reveal the final outcome resulting from the combined interactions of

different factors, but not the contributions of individual factors. For example, real-time gene flow among populations could be examined by adding more markers to the microsatellite dataset, but understanding the phenology of reproductive events (male and female flowering) in natural pinewoods could help in interpreting observed patterns. These processes among pinewoods could vary among years due to differences in the level of temporal climate variation. For instance in Finland, the Finnish Forest Research Institute maintains a country-wide network of sites where phenological events in different tree and plant tree species have been regularly recorded since 1997 (http://www.metla.fi/metinfo/fenologia/index-en.htm).

6.4 Practical implications for pinewood management

The purpose of this study was to examine whether the current seed zones of Scots pine in Scotland reflected patterns of adaptive genetic variation in the species. Collectively, the results of the study show evidence of environment-driven adaptive divergence among native pinewoods across Scotland. This has practical implications because seedling transfers along environmental gradients are known to influence the performance and mortality of the transferred stock (Persson and Ståhl 1990), and because the current management guidelines in Scotland do not take into account the possibility of adaptation to local climates (Ennos, Sinclair et al. 1997; Ennos, Worrell et al. 1998). The purpose of seed zones is to define areas within which seed stock can be moved with limited risks of maladaptation (White, Adams et al. 2007), and zones can have either fixed or floating borders (Ying and Yanchuk 2006 and references therein). From a biological point of view, zones with fixed borders work best when geographically close populations form environmentally homogenous groups and when differences among these groups are large. However, if spatial environmental heterogeneity is high across short geographic distances, the closest neighbouring population might be genetically differentiated, and populations adapted to similar conditions might in fact be located further away. In such cases, it would be best to match the environments of the planting site and the origin of seed stock.

Because this study was carried out under common-garden conditions, there are no data on the effects of seed transfers along environmental gradients in nature, or on the level of local adaptation (i.e., if the local provenance always performs 'best' at its home site). Also, the climate data should be approached with caution as they are heavily based on interpolation and their current precision is only 5×5 km (Perry and Hollis 2005), meaning that actual conditions at the pinewood sites might differ from the grid estimates. An alternative approach is to use Ecological Site Classification (Pyatt, Ray et al. 2001) which allows site-specific estimates of accumulated temperature and moisture deficit based on given latitude, longitude, and altitude. However, these estimates are also based on interpolation and it is not clear which method gives the most accurate estimates. If the current state of native pinewoods is considered natural (it is assumed that trees are adapted to the conditions at their home sites) and if the long-term conservation goal is to maintain them in that state but also to improve their adaptive potential, the recommendation on sourcing seed stock is the most natural – and most restrictive – option: promoting natural regeneration in all populations and when planting seedlings, using seed stock from a range of parents from the same population. Similar general guidelines for different plant species have been proposed by McKay, Christian et al. (2005). In cases where no local stock is available and in very small populations with a limited number of trees and poor-quality seed, stock from other, larger and environmentally similar sites should be used. For this approach to work, it is essential that extensive environmental data are compiled to create site-specific environment profiles that could then be matched using software designed for this purpose. In British Columbia, floating seed zones are widely used (Ying and Yanchuk 2006), and in Sweden, a web-based software, 'Planter's tool', is used to match a planting site with suitable seed orchards ranked according to the product of height growth and survival (Hannerz and Ericsson 2007). Note that the approach proposed for Scotland does not aim at improving tree quality, i.e., increasing the growth while keeping mortality as low as possible. From nature's point of view, it is insignificant whether a natural population grows best at its home site or somewhere else. Instead, it aims to provide material from sites which best match the conditions at the planting site.

The 'local only' approach is sometimes considered risky, especially when dealing with economically important species, because climate change is expected to change environmental conditions across wide areas, thereby changing the optimum fitness and resulting in local populations growing in conditions they are not properly adapted to (e.g. St. Clair and Howe 2007). For a population to track its optimum, adaptive genetic variation in traits under selection is required (Falconer and Mackay 1996), but it has been suggested that in trees, the response to a changing environment could be very slow due to their longevity and to lack of available space (Savolainen, Bokma et al. 2004). However, lack of genetic variation is unlikely to be a major problem in most forest tree populations, as a high proportion of total phenotypic variation is often found within populations (e.g. Howe, Aitken et al. 2003). Thus, adaptive genetic diversity in seed stock can be maintained even when sampling seed from only one population. Molecular marker studies on Scottish populations have shown high levels of diversity within and only small differences among populations (Forrest 1980; Kinloch, Westfall et al. 1986; Provan, Soranzo et al. 1998) which points to significant historic gene flow across Scotland. No data are yet available on the real-time dynamics of gene flow. Based on the mating system analysis, it is likely that outcrossing rates are high in most Scottish pinewoods, and models based on data from Spanish Scots pine populations indicate that outcrossing rates can remain high even in small populations (~15 ha) if tree density is high enough (min. 20/ha, Robledo-Arnuncio, Alía et al. 2004). In populations that consist of a limited number of trees spread over a wide area, rates of selfing and bi-parental inbreeding might be higher which might decrease their potential to evolve. In such cases, introductions of seedlings from other environmentally similar locations can be beneficial.

The molecular genetic basis of quantitative trait variation is being actively investigated in trees and in Scots pine (García-Gil, Mikkonen et al. 2003; Wachowiak, Balk et al. 2009; Wachowiak, Salmela et al. 2011), and based on current data, it appears likely that many loci, each with small effects, control complex trait variation. Theory predicts that in species with strong gene flow, extensive phenotypic differentiation among populations can occur via minor parallel allele frequency changes at underlying loci, resulting in the covariance (linkage disequilibrium)

among alleles in different populations accounting for a large proportion of the phenotypic variation (Latta 1998; Le Corre and Kremer 2003). Recently, evidence of such a pattern has been documented in photoperiodic pathways genes of *Populus tremula* in Sweden (Ma, Hall et al. 2010). If this is a general pattern across complex traits, adaptation from standing variation might be possible which is expected to lead to faster evolution compared to cases where evolution depends on an introduction of new mutations (Barrett and Schluter 2008). Evidence of long-term selection acting on genetic variation already present has also been documented in lab populations of Drosophila (Burke, Dunham et al. 2010). However, in the case of Scots pine, evolution will also depend on new seedlings becoming established at their home sites, and this process can be hindered, for instance, by lack of space (Savolainen, Bokma et al. 2004), other vegetation, and grazing pressure by deer and livestock (e.g. Scott, Welch et al. 2000). If natural regeneration is slowed down by the presence of adult trees, creating open spaces (Kuparinen, Savolainen et al. 2010) and shortening the interval between recruitment events (Kramer, Buiteveld et al. 2008) could be used to facilitate adaptation via natural regeneration. For successful seedling recruitment it is essential that regenerated areas are protected from grazing pressure and that planting at inappropriate sites is avoided.

Assisted migration has also been suggested as one way of maintaining the adaptive potential of natural populations (Hoegh-Guldberg, Hughes et al. 2008; Kramer and Havens 2009). Using this approach, seedlings that currently grow in conditions similar to those predicted at a site in the future would be used. While undeniably an attractive strategy, especially from a tree breeder's point of view, assisted migration is a very risky method that is based on predictions about future climate and on an assumption that some populations already exist that are adapted to those conditions. For example, it has been suggested that in Douglas-fir, current lower altitude or latitude populations could be transferred further north or higher up in altitude so that the transferred stock would be better adapted to future climate (St. Clair and Howe 2007). Climate change scenarios have been developed for Scotland, and temperature increase, drier summers, and wetter winters are expected (Ray 2008). However, there is no guarantee that these factors will be main drivers of adaptation in the future and

additional factors such as the frequency of extreme weather events, changed interactions between species, and the spread of pests and pathogens (e.g. Parmesan 2006; Richardson, Rundel et al. 2007) could also have major influence.

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8. Supplementary material

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