GENETIC BASIS OF ADAPTATION: BUD SET DATE AND FROST HARDINESS VARIATION IN SCOTS PINE

PÄIVI HURME

Department of Biology

OULU 1999

PÄIVI HURME

GENETIC BASIS OF ADAPTATION: BUD SET DATE AND FROST HARDINESS VARIATION IN SCOTS PINE

Academic Dissertation to be presented with the assent of the Faculty of Science, University of Oulu, for public discussion in Kuusamonsali (Auditorium YB 210), Linnanmaa, on January 14th, 2000, at 12 noon.

OULUN YLIOPISTO, OULU 2000

Manuscript received 20 December 1999
Accepted 21 December 1999

Communicated by
Professor W.T. Adams
Professor Robert Podolsky

ISBN 951-42-5504-6

ALSO AVAILABLE IN PRINTED FORMAT

ISBN 951-42-5503-8
ISSN 0355-3191 (URL: http://herkules.oulu.fi/issn03553191/)

OULU UNIVERSITY LIBRARY
OULU 2000

Hurme, Päivi, Genetic basis of adaptation: bud set date and frost hardiness variation in Scots pine

Department of Biology, P.O. Box 3000, FIN-90014 University of Oulu, Oulu, Finland

1999

Oulu, Finland

(Manuscript received 20 December 1999)

Abstract

The genetic basis of large adaptive differences in timing of bud set and frost hardiness between natural populations of Scots pine (*Pinus sylvestris* L.) was studied with the aid of RAPD markers and quantitative genetic tools. Steep clinal variation was found for both traits among Finnish Scots pine populations, and the differences between populations were found to be largely genetic. QTL mapping with Bayesian analysis revealed four potential QTLs for timing of bud set, and seven for frost hardiness. The QTLs were mostly different between the two traits. The potential QTLs included loci with large effects, and additionally smaller QTLs. The largest QTLs for bud set date accounted for about a fourth of the mean difference between populations. Thus, natural selection during adaptation has resulted in fixation of genes of large effect. This result is in conflict with the classical infinitesimal model, but agrees with the results of ORR (1998), suggesting fixation of large effects during adaptation.

The applicability of RAPD and SSCP markers in quantitative genetic studies was also studied. The SSCP technique was found to be efficient in finding polymorphic markers. SSCP polymorphism in coding genes may provide candidate genes for QTL mapping studies. RAPDs were found to be useful for many descriptive analyses, but specific analyses would require more caution.

Keywords: adaptation, Scots pine, QTL mapping, molecular markers, *Pinus sylvestris* L.

Acknowledgements

I thank my supervisor Prof. Outi Savolainen for providing an interesting subject of research and for guidance during this work. I thank Prof. Seppo Lakovaara, the head of the Department of the Biology, for providing good working facilities in the department. I thank Dr. Antoine Kremer for the opportunity to visit INRA in Cestas, France. I also want to thank my coauthors, Tapani Repo, Timo Pääkkönen, Mikko Sillanpää, Elja Arjas, Claus Vogl and Christophe Plomion for pleasant and rewarding collaboration. I thank Prof. W. T. Adams and Prof. Robert Podolsky for comments on my thesis. I thank Anne Karjalainen, Virpi Leskelä and Hannele Parkkinen for their effective and reliable technical assistance in the laboratory. For growing pine seedlings, I thank Teijo Nikkanen, Esko Oksa (The Finnish Forest Research Institute, Punkaharju Research Station) and Ville Pirttilä (Haapastensyrjä Tree Breeding Centre) for advice and help in obtaining the seed material, and Jouko Lehto, Maija Rinkinen and Sakari Silvennoinen (The Finnish Forest Research Institute, Punkaharju Research Station) for their technical assistance in the greenhouse. I also thank the Finnish Forest Research Institute, Punkaharju Research Station and Haapastensyrjä Tree Breeding Centre for supplying the seed material, and the greenhouse facilities. I thank Aila Lindroth and Marjaana Hynninen for their assistance in measuring seedling heights in Punkaharju. I thank the present and former colleagues of the Plant Genetics group: Helene Freville, Elena Baena Gonzales, Anita deHaan, Vladimir Dvornik, Anni Harju, Auli Karhu, Matti Karjalainen, Päivi Karvonen, Hans Koelewijn, Helmi Kuittinen, Katri Kärkkäinen, Merja Mikkonen, Sami Oikarinen, Nina Pitkänen, Robert Podolsky, Mona-Anitta Riihimäki, Anu Sirviö, Rob vanTreuren, Claus Vogl, and Jaana Vuosku for pleasant collaboration. I also thank my parents and my brother Petri for support and company during the work. I especially thank my husband Jari for sharing all things with me, whether work was going fine or not.

Finally, I thank the Research Council for Agriculture and Forestry/Environment and Natural Resources (through funding to Outi Savolainen) and the Ministry of Education (Graduate school of evolutionary ecology) for financially supporting this work.

Oulu, December 1999 Päivi Hurme

Abbreviations

cM	centiMorgan, map distance unit
MAS	Marker assisted selection
PCR	Polymerase chain reaction
QTL	Quantitative trait locus
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SSCP	Single strand conformation polymorphism

List of original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Hurme P, Repo T, Savolainen O & Pääkkönen T (1997) Climatic adaptation of bud set and frost hardiness in Scots pine (*Pinus sylvestris*). Can J For Res 27: 716-723.

II Savolainen O, Hurme P & Repo T (1999) High genetic differentiation of bud set date and frost hardiness in *Pinus sylvestris*. (submitted to Heredity).

III Hurme P & Savolainen O (1999) Comparison of homology and linkage of random amplified polymorphic DNA (RAPD) markers between individual trees of Scots pine (*Pinus sylvestris* L.). Mol Ecol 8: 15-22.

IV Plomion C, Hurme P, Frigerio J-M, Ridolfi M, Pot D, Pionneau C, Avila C, Gallardo F, David H, Neutelings G, Campbell M, Canovas FM, Savolainen O, Bodenes C & Kremer A (1999) Developing SSCP markers in two *Pinus* species. Mol Breed 5: 21-31.

V Hurme P, Sillanpää MJ, Arjas E, Repo T & Savolainen O (1999) Adaptation in Scots pine is based on alleles with large effect on bud set date and frost hardiness. (submitted to Genetics)

Contents

1. Introduction

Life of plants is hard in northern environments, and many adaptations are required to survive and reproduce. The warm season is short, with less than 150 days of thermal growing season in many areas. Growth and reproduction must coincide with favorable conditions. After the period of growing, plants must survive the cold season, during which the temperature may decrease to between -40 and -60 °C. Mechanisms required for acclimation and survival include cessation of metabolic and growth processes, formation of resting buds and enhancement of cold tolerance (see refs. in Savolainen & Hurme 1997).

Scots pine (*Pinus sylvestris* L.) is a major conifer species across the northern boreal zone in Europe and Asia. It has the widest distribution of all pine species, from Spain (38°N) in the south to northern Finland (68°N), and from western Scotland (6°W) to eastern Siberia (135°E) (Mirov 1967). The environmental gradient is steep even when just comparing southern and northern Finland: the length of the thermal growing season (days with average temperature above 5 °C) is about 170 days at latitude 60°N (southern Finland) and less than 120 days at latitude 69°N (northern Finland). Scots pine colonized these areas about 10 000 years ago, after glaciation. This has required a major adaptive shift in relation to the growing season. During a warm period, between 7000 and 5000 B.P., the distribution extended even further to the north of the current northern limit (Hyvärinen 1987).

It is evident that there is a dramatic increase of stress caused by severe climatic conditions between these latitudes (Sarvas 1972, Eriksson *et al.* 1980, Luomajoki 1993) in Finland, resulting in, e.g., increasing mortality (Eriksson *et al*. 1980) and decreasing probability of seed maturation (Henttonen *et al*. 1986) and seed set (Koski & Tallqvist 1978) towards the north. Adaptation of Scots pine populations across these areas has led to population differentiation in many growth related traits (see refs. in Savolainen & Hurme 1997). Reciprocal transfer experiments indicate that the differences have a genetical basis. When southern trees are transferred to the north their survival is reduced (Eiche 1966, Eriksson *et al.* 1980, Beuker 1994). Northern trees in the south have increased growth and survival, but their growth is still slower than that of the local southern populations (Beuker 1994).

Differences in adaptive traits are likely to be due to natural selection, since efficient pollen migration between latitudes (Koski 1970) opposes differentiation by genetic drift. This can be seen at neutral marker loci, where differentiation between northern and southern Finland is close to zero (F_{ST} 0.02) (Karhu *et al*. 1996). Practically infinite population size of Scots pine also suggests that adaptive differentiation is indeed caused by natural selection, and not by population substructure or drift.

1.1. Genetic basis of adaptive variation

Many traits related to adaptation and fitness are assumed to be influenced by many genes (polygenes), and are called quantitative traits. Segregation of many genes results in a continuum of phenotypes in a population. In natural populations, the distribution of phenotypes is approximately Gaussian (normal distribution), average phenotypes being the most abundant and extremes less frequent.

The details of the genetic basis of adaptive variation in natural population are still largely unknown. Fisher (1930) suggested that many genes with additive, small effects contribute to quantitative traits. He also proposed that this same model would account for adaptive evolution, under strong natural selection. Later Kimura (1983) and Orr (1998) showed that the mutations fixed during an adaptive process due to directional selection are not expected to be uniformly small, but to follow an exponential distribution. Existing studies also provided evidence that many adaptations could be based even on single loci. However, the experiments were often on domesticated species, where artificial selection may have fixed genes with major effects between lines (see refs. in Tanksley 1993, Lynch & Walsh 1998). Thus, the results cannot be applied to natural populations. In the few studies to date on natural populations, moderately large QTL effects were revealed for frost tolerance in *Eucalyptus nitens* (Byrne *et al*. 1997), and large effects for flowering time in *Arabidopsis thaliana*, (Clarke *et al*. 1995, Mitchell-Olds 1996, Kuittinen *et al*. 1997) and for bud flush in a cross between *Populus* species (Bradshaw & Stettler 1995). Ongoing studies are listed in a recent review of QTL mapping in trees (Sewell & Neale 1999). Despite the advances, only further empirical studies on natural populations will answer whether few genes with large effects or many genes with small effects underlie genetic variation in adaptive traits.

1.2. Analysis of quantitative trait variation

When studying adaptive traits, preliminarily, information on the general pattern of trait variation is needed. The amount and distribution of variation between and within populations can be determined in population experiments, when the material is grown in a common environment. Further, family studies reveal the amount of genetic differentiation (Q_{ST}) between populations (Prout & Barker 1993). On the basis of family studies also the amount of additive genetic variance, and the heritability can be estimated (Falconer & Mackay 1996).

The detailed analysis of quantitative traits, however, requires identifying individual genes influencing the trait. Polygenic inheritance complicates such analysis, since the number and effects of individual genes cannot be revealed with simple Mendelian analysis.

Statistical methods can be used to estimate numbers of genes affecting the traits from means and variances of crosses between differing lines (Wright-Castle formula in Castle 1921, Lande 1981, Cockerham 1986). Estimates of average gene action and interactions between genes and the environment are gained as well. However, individual loci cannot be detected. Estimates are based on assumptions of complete additivity, equal effects of all genes, no linkage and fixed differences between parental lines, that are not likely to hold true, resulting in minimum estimates of numbers of genes.

Mapping quantitative traits relative to genetic markers (QTL mapping) gives more detailed information on individual genes underlying phenotypic variation. Abundance of various genetic markers nowadays makes the analyses feasible. Co-segregation of markers and phenotypes can be studied in segregating progenies derived from parental lines. Linkage disequilibrium necessary for QTL mapping is obtained by crossing parents differing with respect to the trait. QTL analysis can provide an estimate of the numbers of genes, gene effects and mode of action (additivity, dominance) of individual genes as well as linkage and position in relation to markers. The simplest method, called single marker analysis, is based on comparison of differences between phenotypic means with different allelic constitutions at each marker (Sax 1923). This analysis is coarse, however, and provides no information on recombination between QTLs and markers.

More advanced methods that use information on flanking markers also test the intervals between markers for the presence of QTLs. The first method based on this was interval mapping (Lander & Botstein 1989), followed by regression methods (Jansen 1992, Kearsey & Hyne 1994). Combination of interval mapping and multiple regression facilitated the analysis of multiple QTLs simultaneously (Zeng 1993, 1994, Jansen 1993, Jansen & Stam 1994). The most recent methods for QTL mapping are based on Bayesian theory (Satagopan *et al*. 1996, Uimari *et al*. 1996, Uimari & Hoeschele 1997, Sillanpää & Arjas 1998, Sillanpää & Arjas 1999), where parameters are treated as random variables, and the data are used to update information about these parameters. In other words, prior information of the parameters is used to obtain posterior distribution for these parameters (Weir 1996). This is in contrast to the classical (frequentist) statistics, where data are used to estimate unique values for parameters.

Initially, the QTL mapping methods were mainly for inbred line crosses where limited numbers of alleles are segregating between genetically fixed lines. In outcrossing species, however, the fixation of different alleles between populations can not be expected, and many alleles may segregate within and between populations. This also causes uncertainty in inferring haplotypic arrangements in the crossing experiments. Methods for outcrossing species have been developed for full sib or half sib arrangements. The full sib methods are generally for F_2 designs, and require genotypic information from grandparents (Beckmann & Soller 1988, Haley *et al.* 1994, Knott *et al.* 1997) or known haplotypes from parents (Maliepaard & Van Ooijen 1994, Jansen 1996). If this information is not available, it is possible to apply methods developed by Jansen *et al.* (1998) and Sillanpää and Arjas (1999), which do not require either of these.

A few methods for half sib designs have been developed, as well. Georges *et al*. (1995) and Knott *et al.* (1996) presented analyses for half sib family structures typical in livestock species. A composite interval mapping method of Wu (1999), on the other hand, is designed for conifers, in which the haplotypes of the progeny are obtained directly from the megagametophytic tissue. In this method, the outcrossing rate and QTL genotype frequencies in the pollen pool are estimated from the data. However, in Scots pine, the outcrossing rate is close to 100 % (Muona & Harju 1989).

1.3. Bud set

Terminal bud set is one of the adaptive requirements of Scots pine trees in the autumn, since the right timing of bud set is essential for survival. Before the onset of low temperatures, growth ceases, and is followed by formation of resting buds that contain primordia for the next growing season. In first-year seedlings, bud set occurs after a free period of growth, whereas later primordia of the resting buds predetermine the growth for the next growing seasons (Jablanczy 1971, Lanner 1976). However, approximately similar timing of the events in the juvenile and adult stages between populations can be expected (Cannell & Willet 1975, Ununger *et al.* 1988, Ekberg *et al*. 1994).

Latitudinal differences in the length of the growing season have caused selection that has differentiated populations with respect to bud set. In the north, bud set occurs earlier than in the south. Differences are also apparent when populations are grown in the same environment (common garden experiments), indicating genetic differences between populations (Mikola 1982, Karhu *et al*. 1996). Several genes have been suggested to influence bud set (Mikola 1982). Variation within populations was not studied by Mikola, but the long time span of bud set within populations indicates that it is extensive as well.

1.4. Frost hardiness

Frost hardening is important for survival over the cold season. Hardening starts as a response to declining day length and temperature, but their exact roles in the process are not thoroughly understood (Ekberg *et al.* 1979, Eriksson *et al*. 1978, Koski & Sievänen 1985, Hänninen *et al*. 1990). Frost hardiness, like bud set, shows a latitudinal cline, so that northern populations of Scots pine start hardening earlier than southern populations (Hagner 1970, Toivonen *et al*. 1991, Aho 1994). This has been reported also in other conifer species, e.g. in *Picea abies* (Ekberg *et al*. 1979) and *Pinus contorta* (Hagner 1970, Jonsson *et al*. 1981). Studies have been based primarily on between population variation, but within-population variation in frost hardiness is extensive as well (Aitken & Adams 1996, Aitken *et al.* 1996).

For frost tolerance, many candidate genes are available, especially for crop species, but there are also some for conifers, e.g. one dehydrin gene for *Pseudotsuga menziesii* (Jarvis *et al*. 1996) and chitinase genes (antifreeze-like proteins) in *Pinus* species (Chang *et al*. 1996; Wu *et al*. 1997).

1.5. Molecular markers

A prerequisite for marker development and marker mapping is variability in DNA sequences between individuals and species. The most widely used markers currently are based on the amplification of DNA sequences with polymerase chain reaction (PCR) (Mullis & Faloona 1987). With PCR, sequences from arbitrary and specific DNA regions can be obtained.

Arbitrary markers are relatively easily obtained, since former information on the DNA sequences is not needed. In the RAPD technique, random regions of DNA are amplified with short arbitrary primers (Williams *et al.* 1990, Welsh & McClelland 1990), which either amplify a specific region (+ allele) or not (- allele). The amplification patterns are revealed by electrophoresis on a gel. In diploid tissue, the dominant homozygote (++) cannot be distinguished from the heterozygote (+-), which lowers the information content of RAPDs. In conifers, dominance is not a problem, since the markers can be scored directly from the haploid megagametophytes of a single tree (Carlson *et al.* 1991).

More information can be obtained from codominant markers where each genotype can be scored. The most feasible future markers may be those obtained by amplification of genes with known function, as they can be used in comparative mapping studies or as candidate genes in QTL mapping studies. Efficient methods to find polymorphisms in such sequences have been developed. The SSCP (single strand conformation polymorphism) technique is based on conformational structures of DNA sequences in specific gel conditions (Orita *et al.* 1989a,b). This sensitive method can detect differences of even one base pair between alleles. DNA is denatured into a single-stranded form, and the strands are separated on the non-denaturing polyacrylamide gel. Separation is based on the conformational differences of single-stranded DNA fragments that are detected as mobility shifts. Strands of the same allele usually have different mobilities and thus can be seen as two bands on the gel. SSCP has been shown to be useful for detecting polymorphism in PCR products of large size in plants (Bodénès *et al.* 1996). More sensitivity can be obtained by using different temperatures during the electrophoresis (Glavac & Dean 1993), or by adding substances, like glycerol to the gel (Hayashi & Yandell 1993).

1.6. Goals of this work

The aim of this study was to examine the genetic basis of large adaptive differences in bud set timing and frost hardiness in natural populations of Scots pine, and to evaluate the results in the light of theories on adaptive evolution. The high differentiation in bud set timing and development of frost hardiness between northern and southern Finland provided the starting point of the studies. First, we studied clinal variation of the traits in populations, and their inheritance in half sib families. To understand the genetic basis in more detail, information on the number of genes and their relative effects were needed. We searched for genes responsible for variation in bud set date and frost hardiness with QTL mapping, and located the genes on a RAPD marker map. We also studied the applicability of RAPD and SSCP markers in quantitative genetic studies.

2. Materials and methods

2.1. Scots pine material

2.1.1. Seedling production

To study the genetics of bud set date and frost hardiness (I, II, V), we grew seedlings of Scots pine in a greenhouse in Punkaharju (Finnish Forest Research Institute, Punkaharju Forest Research Station), and observed the traits after the first growing season. Latitudinal variation of bud set date and frost hardening in Finland provided the starting point for the studies.

Geographical variation of the traits was examined by growing population samples from different parts of the cline (60-67 °N) in Finland (I, V). The populations used in this study were Salla, Sotkamo (northern Finland), Kerimäki and Bromarv (southern Finland).

Together with the population samples, backcross progenies originating from crosses between northern and southern Finland were grown (I, V). These plants were used to study the inheritance of the trait variation, and to map genes influencing bud set date and frost hardiness. All the progenies grown were ‘open pollinated backcrosses’ (half sib progenies) obtained in a following way. Trees of the northern origin had been crossed with southern trees, and the F_1 trees had been planted in southern Finland. Open pollinated seeds were collected from the trees regarding them as backcrosses to surrounding southern pollen. In the QTL mapping experiment (V), the backcross progeny used originated from a single F_1 tree from a cross between plus trees P315 (Kemijärvi, northern Finland) and E1101 (Kerimäki, southern Finland).

Half sib families were grown to study the heritability of bud set date and frost hardiness (II). The material came from Kolari, northern Finland, and Lapinjärvi, southern Finland (the standard forests of the Finnish Forest Research Institute). The seeds were from individual trees, and since the outcrossing level of Scots pine is about 0.95 (Muona & Harju 1989), we assumed seeds from a single tree to be half sibs.

2.1.2. Material for RAPD homology comparisons

For the study of comparison of homology of RAPD markers (III), we used haploid megagametophytic DNA from three trees. These were the plus trees P304 (north) and E1101 (south) of the Finnish Forest Research Institute, and an F_1 tree originating from a cross between P315 (north) and E1101 (south).

2.1.3. Progeny for SSCP markers

We developed SSCP markers for two *Pinus* species, Scots pine and Maritime pine (*Pinus pinaster* Ait.) (IV). For Scots pine, we used F_1 progeny from a cross between trees E635C and E1101 (both from southern Finland). Diploid DNA from the needles of 48 F_1 trees and the two parent trees was used. For Maritime pine, we used a three generation inbred pedigree (Plomion *et al*. 1995). The family consisted of two grandparents (accessions L146 and C10), one hybrid parent (H12) and 62 megagametophytes collected from F_2 seeds of H12.

2.2. Bud set

First year pine seedlings, grown in the greenhouse in southern Finland, will set an easily visible terminal bud towards the end of the summer. The timing of terminal bud set was scored twice a week from the beginning of August until October (I, II, V). The bud was regarded as formed when it was seen clearly between the needles above the seedling. The date of bud set was scored from each seedling and was given as the number of days from sowing to the date of bud set.

2.3. Frost hardiness

Frost hardiness measurements were done based on freezing treatments in air-cooled chambers. First, frost hardiness was studied at the population level (population level estimates) at two-week intervals during the hardening period (I). Each time different frost temperatures were used to induce different degrees of damage. The damage of the seedlings after freezing treatment was assessed by three methods: 1) by the electrolyte leakage method on needles, 2) by visual scoring on needles, and 3) by impedance analysis as extracellular resistance on stems (Repo *et al*. 1994).

For heritability estimation and QTL mapping, frost hardiness measurements on individual seedlings were made, based on visual scoring of frost damage on needles (II, V). We exposed the seedlings to a pre-selected treatment temperature (LT_{50}, causing intermediate frost damage). After frost treatments, the seedlings were kept in a greenhouse in the warm and long day conditions, and after 10 days, the damage to the seedlings was assessed on a visual scale of 0 to 10.

2.4. Quantitative trait analysis

2.4.1. Population variation in bud set date and frost hardiness

Population samples from different latitudes in Finland were used to study geographical variation of bud set date and frost hardiness (I). The general pattern of clinal variation during the growing season was studied by examining the distribution of variation between populations. We also studied the phenotypic association between the traits at the population level.

2.4.2. Inheritance of bud set date and frost hardiness

We studied the inheritance of bud set date and frost hardiness in the half sib families (II). For this, the additive genetic variances and heritabilities were determined (Falconer & Mackay 1996). We also estimated the amount of genetic differentiation (Q_{ST}) between northern (Kolari) and southern (Lapinjärvi) populations (Prout & Barker 1993). To examine the potential genetic association between bud set and frost hardiness, we estimated genetic correlation (independent vs. pleiotropic genes) between the traits (Falconer & Mackay 1996).

2.5. Molecular markers

2.5.1. RAPD markers

RAPDs were assayed on haploid megagametophytes (nutritive tissue of the seeds) of single trees to avoid the problem of dominance. Usually megagametophytes can be obtained directly from collected seeds of the trees (III). In our QTL mapping experiment (V), the backcross seeds had to be grown into seedlings, and the megagametophytes were collected off the germinated seedlings for DNA isolation. This was possible, since in conifers, the seed coat with the megagametophyte resides on top of the seedling after germination. The megagametophyte has the same genotype as the egg cell, and in this way, we were able to assess the genotypes of the female gametes produced by the heterozygous F_1 maternal tree.

In comparing the homology of RAPD markers (III), restriction with endonucleases, and linkage analysis were used for verifying homology between similar sized bands between trees. Similar restriction patterns between bands were considered as evidence for homology. Linkage analysis of heterozygous (segregating) markers was further used to confirm homology vs. non homology.

2.5.2. SSCP markers

For SSCPs, both the haploid megagametophytes and the diploid needle tissue were used (IV). We searched the gene bank for nuclear sequences; cDNA or genomic clones of genes with known function, or sequences identified on the basis of similarity to known genes (EST sequences). Multicopy gene families, common in pines, were avoided. Primers were designed to amplify parts of genes, and if intron positions were available, they were included in the amplified fragment. After PCR, the amplified fragments were run on the non-denaturing gel (SSCP analysis) in different electrophoretic conditions (variable temperature and voltage / cm) to find segregating markers.

2.6. Mapping

2.6.1. Construction of a RAPD map

We used RAPD markers as genetic markers in QTL mapping (V). We constructed a RAPD map from 84 random megagametophytes collected off the germinated backcross seedlings originating from a single F_1 tree (see sections 2.1.1 and 2.5.1.). Loci with the megagametophyte genotypes segregating 1:1 in the backcross progeny were chosen for mapping. The RAPD map was constructed with Mapmaker / Exp 3.0 (Lander *et al*. 1987) using the Haldane mapping function. First, the markers were divided into linkage groups, and then their relative orders in the groups were determined with a multi-point analysis. All potential scoring errors were verified, scoring uncertain double recombinants as missing data.

2.6.2. QTL mapping

We searched for the genes influencing bud set date and frost hardiness with QTL mapping and located them in relation to the markers in the RAPD map (V). Growing of the backcross progeny seedlings was conducted twice, first in 1994 and the second time in 1996. In 1994 only bud set was studied, and in 1996 both bud set and frost hardiness. The bud set data sets from 1994 and 1996 were also analyzed together to verify the results obtained for separate data sets.

In our crossing arrangement, we studied the maternal component only. We had no marker information available from the grandparents (P315 or E1101) or the father (southern pollen). Thus, we applied a Bayesian QTL mapping method developed previously for outcrossed offspring by Sillanpää & Arjas (1999), with specific modifications for our design. For simplicity, constant QTL effects from the father’s side were assumed.

After scoring bud set and frost damage in the progeny, the seedlings from the tails of the phenotypic distributions were chosen for genotyping and QTL mapping. Such selective genotyping improves the power of QTL detection (Lander & Botstein 1989, Darvasi & Soller 1992, Tanksley 1993).

First, markers were tested separately for association with QTLs with a single marker analysis. The most significant markers obtained from this were then chosen as background controls in the Bayesian QTL analysis. Then, the Bayesian analysis was performed, and the potential QTLs segregating in the cross were obtained from the posterior distribution of QTL intensities in the analysis. Once the potential QTLs were found, the individual QTL effects were calculated from random sets of individuals. We calculated the proportions of phenotypic and genetic variance explained by the QTLs. Finally, the results were examined in the evolutionary and breeding context.

3. Results and discussion

3.1. Population differentiation in bud set date and frost hardiness

A latitudinal cline was found in timing of bud set in Finnish populations of first year seedlings (I). The populations from northern Finland set buds earlier than the populations from southern Finland. In 1994, the difference in the median date of bud set between the northernmost (Salla) and the southernmost (Bromarv) population was about three weeks. Of the total variance, 36 % was between the four populations. In 1996, bud set in the populations took place in the same order, but the differences between populations were larger. Bud set took place 38 days earlier in Salla than in Bromarv, and of the total variance, 76 % was between populations. Clinal variation here corresponds with the results of Mikola (1982), and has been observed in growth related traits in many other temperate tree species of the Northern Hemisphere, as well (e.g. Heide 1974, Skrøppa 1982). Vaartaja (1959) found photoperiodic ecotypes of growth rhythm in 38 tree species.

Latitudinal variation was found for frost hardiness as well: northern populations started hardening earlier than the southern ones. Similar findings have been reported earlier in Scots pine (Hagner 1970, Toivonen *et al.* 1991, Aho 1994), and e.g. in *Picea abies* (Ekberg *et al*. 1979) and *Pinus contorta* (Hagner 1970, Jonsson *et al*. 1981). These findings emphasize the general pattern of adaptation.

The steep clinal variation found in timing of bud set and frost hardiness is largely based on genetic differences between populations. In the family studies, the proportion of genetic variance between northern (Kolari) and southern (Lapinjärvi) populations (Q_{ST}) was 0.82 (II). As far as we know, this value of Q_{ST} is the highest such estimate in any organism, documenting strong diversifying selection across these latitudes. This is in contrast to the pattern at neutral markers, where differentiation between northern and southern Finland is close to zero for allozymes, RFLPs and microsatellites (F_{ST} 0.014 - 0.02) (Karhu *et al*. 1996) due to extensive pollen flow. Further, because we studied trait variation in seedlings, the level of differentiation between adult trees after selection, could be even higher.

For bud set dates, we could also examine within-population variation, as these were observed for each seedling individually (I). Much variation was found in populations, and the northern population from Salla had at least equal amounts of phenotypic and

presumably genetic variation as the more central populations. High within population variation in the duration of growth is also typical of other northern forest tree species, as in *Picea abies* (Ekberg *et al*. 1985). Certainly, northern populations undergo heavy selection at early stages of life. However, further adaptation of the populations at the northern limit may be impeded by efficient pollen migration from the south.

In the family studies, moderately high heritabilities were obtained: 0.33 and 0.67 in bud set for the southern and the northern population, respectively, and 0.20 and 0.36 in frost hardiness for the populations, respectively (II). However, the standard errors of the estimates were fairly large (0.13 - 0.21). Moderate and high heritabilities have been observed in different ages in *Pseudotsuga menziesii*, as well (Li & Adams 1993). High heritabilities of adaptive traits indicate, that despite the strong selection, much additive genetic variation is left. In our case, migration and the young age of the seedlings may have increased variances, and these could be reduced by selection later in the life cycle.

Examination of the two traits together revealed that the development of frost hardiness started when most of the buds had formed (I). High correlation between the traits at the population level was found (r = 0.69 - 0.97), which agrees with findings in other tree species (e.g. Johnsen & Apeland, 1988). Our results indicated that in the greenhouse conditions used (natural photoperiod and temperature conditions), bud set predicted the initiation of frost hardiness at the population level. The genetic correlation between bud set and frost hardiness was 0.41 (southern population) and 0.57 (northern population) in the family studies, suggesting a partly shared genetic basis of the traits (II). However, the analysis cannot distinguish close linkage and pleiotropic gene effects (Falconer & Mackay 1996), and the actual function of the ‘timing genes’ remains unknown.

3.2. Genetic basis of bud set date and frost hardiness

Our goal was to estimate the number of loci and the size of their effects for bud set date and frost hardiness, and to view the results in the context of adaptive evolution theories (V). Relevant in our data was that we studied natural populations, not influenced by human selection. Further, we studied QTLs responsible for within species differences, and we studied an outcrossing species with practically infinite population size.

The RAPD map constructed contained 164 RAPD markers, distributed in 16 linkage groups: 12 larger groups (from 35 to 136 cM), and 4 smaller (from 2 to 11 cM). The map spanned 1000 cM, covering about half of the genome, estimated with the method of Chakravarti *et al*. (1991).

Altogether, four potential QTLs influencing timing of bud set were found with the Bayesian analysis. Different QTLs were detected in 1994 and 1996. When the data for both years were combined, the QTLs found in separate years were significant, and an additional QTL not found in separate data sets was also detected. The differences between years may be due to interactions between QTLs and the environment (Melchinger *et al.* 1998). However, no significant genotype x environment interactions were found, even though the environments were clearly different between years. Plomion *et al.* (1996), Emebiri *et al.* (1998) and Kaya *et al.* (1999) found different potential QTLs for height

growth at different ages in *Pinus*. This may be due to differential QTL expression during maturation, but may also have been influenced by environmental variation or genotype x environment interaction.

For frost hardiness, seven potential QTLs were found with the Bayesian analysis. These were mostly located in different linkage groups than the bud set QTLs. However, two linkage groups showed QTL activity for both bud set and frost hardiness. In one group they were clearly separated, but they were close to each other in the other group. Otherwise, no genetic association between the traits was found.

All QTLs underlying trait variation still may not have been found due to the specific features of our design. First, fixation of alleles between grandparental populations could not be assumed, and thus all loci influencing the traits may not have been segregating in the cross. Second, we lacked information on the paternal component, and a constant effect from the father's side was assumed. We know, however, that the pollen pool is variable, and this may decrease the power of the analysis. The incomplete coverage of the RAPD map may have also left some QTLs undetected.

The individual QTL effects (percentage of phenotypic variance explained) in our study ranged up to 12.7 % for bud set date, and up to 11.1 % for frost hardiness. Altogether, the markers explained 3.5 and 15.4 % of the total variation in bud set data 1994 and 1996, respectively. For frost hardiness, 24.9 % of the variation was explained by the markers.

For comparison, few studies on natural outcrossing populations, where the differentiation is generated by natural selection, are available. In *Eucalyptus nitens*, two QTLs with effects of 8 and 11 % of the total phenotypic variation were found for frost tolerance (Byrne *et al*. 1997). In natural populations of *Drosophila melanogaster*, variation of bristle number has been suggested to be influenced by alleles with large effect at a few loci (Mackay and Langley 1990, Mackay 1995). A candidate gene, *scabrous*, accounted for 13 and 8 % of the genetic variation in abdominal bristle number, and sternopleural bristle number, respectively (Lai *et al*. 1994), demonstrating the segregation of alleles of large effects in natural populations.

We expected that our effects would be smaller than those found in many QTL studies to date. In domesticated species, artificial selection may fix major genes between lines, and this has been observed in many QTL studies (refs. in Tanksley 1993, Lynch & Walsh 1998). For instance, in barley, frost tolerance loci accounted for 31 or 79 % of the variation in different years (Pan *et al*. 1994). Larger QTL effects were observed also in selfing species, where smaller population effective sizes allow alleles with larger effects to be fixed. This has been reported in *Arabidopsis thaliana*, where flowering time differences between populations were largely due to one major gene and several minor ones (Clarke *et al*. 1995, Mitchell-Olds 1996, Kuittinen *et al*. 1997). In interspecific crosses QTLs fixed may be larger due to longer differentiation, and possible selection for reproductive isolation and QTL differentiation. A cross between *Populus* species revealed five QTLs influencing bud flush, which explained 85 % of the total variation (Bradshaw & Stettler 1995), confirming these expectations.

Further information on the genetic basis of the traits was obtained by relating the QTL effects of our study to the between and within population differences. This was possible on the basis of the half sib study (II). The total variance in a backcross of an F_1 hybrid between a northern and a southern population to the southern parent (V_{BC}) consists of the components ½ V_S, ¼ V_{AN}, ¾ V_{AS} and V_E (Lynch & Walsh 1998, p. 228), where V_S is the

segregating variance between the two populations, and V_{AN} and V_{AS} are the additive genetic variances within northern and southern populations, respectively, and V_E the environmental variance.

The total variation in *within this single* backcross family (V_{WF}) was $\frac{1}{2} V_S + \frac{1}{8} V_{AN} + \frac{5}{8} V_{AS} + V_E$, and the maternal additive genetic contribution to the variance (V_{AM}) *within a single backcross family* was $\frac{1}{2} V_S + \frac{1}{8} V_{AN} + \frac{1}{8} V_{AS}$. Our QTL detection was based on observing marker genes in the maternal component only, and thus the highest proportion of variation that the putative QTLs could account for in the backcross progeny was obtained by dividing V_{AM} by the total expected variance in this backcross (V_{AM} / V_{BC}). Here, the ratio of the estimates $\hat{V}_{AM}$ and $\hat{V}_{BC}$ is 0.6. Thus, the largest QTL for bud set date (13 %), accounts for about 22 % of the maximum possible. Considering the total phenotypic variation explained by all markers, 15.4 % in 1996 bud set data accounts for 26 % of the genetic variance.

If we assume $\hat{V}_{AM} / \hat{V}_{BC}$ to be the same for frost hardiness, about 20 % of the genetic variance is accounted by the largest QTL. Similarly, given that the total phenotypic variance in frost hardiness explained by the markers is 24.9 %, it is estimated that as much as 42 % of the overall genetic variance can be attributed to the QTLs identified in this study.

When relating the size of the largest effect on bud set date to the southern population of half sib families, the additive effect of the QTL (4.5 days between the homozygote and the heterozygote) is equivalent to one additive genetic standard deviation within the southern population. However, we do not know the frequency of the allele in the population, and thus cannot infer the actual contribution to the population variance. Further, the largest QTL alone accounts for a considerable share of the between population difference (mean difference between northern and southern population 27 days). Thus, our results on the size of the largest effects responsible for the adaptive differentiation refute the infinitesimal model: natural selection during adaptation has resulted in the selection of alleles with large effect at least at this one locus. This is in accordance with the theoretical results of Orr (1998).

3.3. Applications of RAPD and SSCP markers

3.3.1. RAPD markers

Comparison of homology of RAPD loci between individual Scots pine trees with restriction analysis revealed, that out of the 33 loci studied, two (6 %) were not homologous to the ones in the reference tree (III). Although the bands were of the same size, and amplified with the same primers, they were representatives of different loci. The two loci were not linked to any markers, which confirmed their nonhomology.

Of the pairwise RAPD marker comparisons, 94 % were homologous in our study, which is about the same magnitude (92 %) as reported in maize (Beaumont *et al*. 1996). Between *Helianthus* species, Rieseberg (1996) reported that 91 % of pairwise

comparisons were homologous. However, more inconsistencies may be expected at the interspecific level, e.g. between *Brassica* species the pairwise homology was only 80 % (Thormann *et al*. 1994).

Experimental errors, often associated with RAPDs, probably caused some inconsistencies in our data, as well. These may be caused by scoring errors, competition in the PCR reactions, low reproducibility, or by co-amplification of different loci with the same mobility.

Altogether, homology problems and experimental errors may lead to problems in some analyses, such as in population genetic analyses, where homology is assumed between similar sized bands (Clark & Lanigan 1993, Lynch & Milligan 1994). Errors may also show up in mapping (e.g. Lin & Ritland 1996), and taxonomic studies (Powell *et al*. 1996). RAPDs are suitable for many general description purposes, but the more specific the question, the more care is needed in analyses. Homology studies would be desirable at least in studies between individuals and crosses, and sometimes even within individuals to verify co-migrating fragments. Often it would be desirable to verify homology between individuals with restriction or linkage analyses.

3.3.2. SSCP markers

The SSCP method was found to be an efficient technique in finding polymorphic loci (IV). Out of the 23 primer pairs used, a single product was amplified from 17 and 18 loci in Scots pine and Maritime pine, respectively. From these, 15 fragments were selected for searching for polymorphism with the SSCP analysis in each species, and polymorphism was found in 11 loci in both species. The ability of the SSCP technique to detect polymorphisms was not affected by the fragment length, which is in contrast to the results in other studies (Hayashi & Yandell 1993, Sheffield *et al*. 1993). The temperature in the run, and the presence vs. absence of intron sequences within the amplified fragment did not affect the number of polymorphisms found, either.

Our markers segregated in a Mendelian fashion in both species, confirming the efficiency of the SSCP analysis in marker development. In Maritime pine, the SSCPs could be located in the marker map generated earlier from the cross. Thus, SSCPs from coding genes can be used to complete maps based on anonymous markers, and also used for comparative mapping studies. These markers will provide valuable candidate genes in QTL mapping.

4. Concluding remarks

In the study of the genetic basis of timing of bud set and frost hardiness, we found that the steep clinal variation in both traits in Finnish Scots pine populations is due to large genetic differences (I, II).

QTL mapping experiment revealed a moderate number of loci for timing of bud set and frost hardiness (four to seven), including loci with large effects, and additionally smaller QTLs (V). The largest QTLs accounted for about a fourth of the mean difference between populations. Thus, natural selection during adaptation has resulted in selection of genes of large effect. This result is in conflict with the classical infinitesimal model, but agrees with the results of Orr (1998), suggesting fixation of large effects during adaptation.

Possible applications of the detailed genetics of quantitative traits include breeding with the aid of markers. Such marker assisted selection (MAS) would make selection at the juvenile stage possible, which would be profitable in forest trees due to their long rotation age (e.g. Neale & Williams 1991, Williams & Neale 1992). MAS will be more efficient than breeding of phenotypes, if the additive genetic variance explained by the markers is larger than the heritability of the trait (Lande & Thompson 1990). The largest QTLs in our study may be large enough to allow breeding with MAS. This would require, however, that the same set of QTLs for bud set date would be expressed in later stages of life cycle. For height growth in *Pinus*, for example, this may not apply, since different QTLs were expressed at different ages (Plomion *et al.* 1996, Emebiri *et al.* 1998, Kaya *et al.* 1999). For efficient breeding, the grandparents should also be genotyped in order to know the origins of the alleles. Otherwise, it is only possible to distinguish them in relation to each other, and deduce the origins from this information.

A useful application of quantitative trait analysis would be the use of candidate genes identified by molecular methods. Such genes could be turned into markers, for example with the SSCP technique, which proved to be powerful in detecting polymorphisms in our study (IV). Potential localization of a QTL in the same region as the candidate gene may indicate that the candidate gene has a role in the trait determination. For frost tolerance, many candidate genes are available, especially for crop species, but some also exist for

conifers, e.g. one dehydrin gene for *Pseudotsuga menziesii* (Jarvis *et al*. 1996) and chitinase genes (antifreeze-like proteins) in *Pinus* species (Chang *et al*. 1996, Wu *et al*. 1997).

RAPDs were used as genetic markers in our QTL mapping study. Although they lack the reliability of better characterized markers, they are useful within specific crosses. Care should be taken, however, in the issues of homology when using these markers in more complicated situations (III). SSCP markers will, on the other hand, make suitable markers for completing maps based on such anonymous markers, and can be used for comparative mapping studies (IV).

5. References

Aho M-L (1994) Autumn frost hardening of one-year-old *Pinus sylvestris* (L.) seedlings: effect of origin and parent trees. Scand J For Res 9: 17-24.

Aitken SN & Adams WT (1996) Genetics of fall and winter cold hardiness of coastal Douglas-fir in Oregon. Can J For Res 26: 1828-1837.

Aitken SN, Adams WT, Schermann N & Fuchigami LH (1996) Family variation for fall cold hardiness in two Washington populations of coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco). For Ecol Manag 80: 187-195.

Beaumont VH, Mantet J, Rocheford TR & Widholm JM (1996) Comparison of RAPD and RFLP markers for mapping F_2 generations in maize (*Zea mays* L). Theor Appl Genet 93: 606-612.

Beckmann JS & Soller M (1988) Detection of linkage between marker loci and loci affecting quantitative traits in crosses between segregating populations. Theor Appl Genet 76: 228-236.

Beuker E (1994) Long-term effects of temperature on the wood production of *Pinus sylvestris* L. and *Picea abies* (L.) Karst. in old provenance experiments. Scand J For Res 9: 34-45.

Bodénès C, Laigret F & Kremer A (1996) Inheritance and molecular variations of PCR-SSCP fragments in Pedunculate oak (*Quercus robur* L.). Theor Appl Genet 93: 348-354.

Bradshaw HD Jr & Stettler RF (1995) Molecular genetics of growth and development in *Populus*. IV. Mapping QTLs with large effects on growth, form, and phenology traits in a forest tree. Genetics 139: 963-973.

Byrne M, Murrel JC, Owen JV, Williams ER & Moran GF (1997) Mapping of quantitative trait loci influencing frost tolerance in *Eucalyptus nitens*. Theor Appl Genet 95: 975-979.

Cannell MGR & Willet SC (1975) Rates and times at which needles are initiated in buds on differing provenances of *Pinus contorta* and *Picea sitchensis* in Scotland. Can J For Res 5: 367-380.

Carlson JE, Tulsieram LK, Glaubitz JC, Luk VWK, Kauffeldt C & Rutledge R (1991) Segregation of random amplified DNA markers in F_1 progeny of conifers. Theor Appl Genet 83: 194-200.

Castle WE (1921) An improved method of estimating the number of genetic factors concerned in cases of blending inheritance. Science 54: 223.

Chakravarti A, Lasher LK & Reefer JE (1991) A maximum likelihood method for estimating genome length using genetic linkage data. Genetics 128: 175-182.

Chang S, Puryear J, Funkhouser EA, Newton RJ & Cairney J (1996) Cloning of a cDNA for a chitinase homologue which lacks chitin-binding sites and is down-regulated by water stress and wounding. Plant Mol Biol 31: 693-699.

Clark AG & Lanigan CMS (1993) Prospects of estimating nucleotide divergence with RAPDs. Mol Biol Evol 10: 1096-1111.

Clarke JH, Mithen R, Brown JK & Dean C (1995) QTL analysis of flowering time in *Arabidopsis thaliana*. Mol Gen Genet 248: 278-286.

Cockerham CC (1986) Modifications in estimating the number of genes for a quantitative character. Genetics 114: 659-664.

Darvasi A & Soller M (1992) Selective genotyping for determination of linkage between a marker locus and a quantitative trait locus. Theor Appl Genet 85: 353-359.

Eiche V (1966) Cold damage and plant mortality in experimental provenance plantations with Scots pine in northern Sweden. Stud For Suec 36: 1-218.

Ekberg I, Eriksson G & Dormling I (1979) Photoperiodic reactions in conifer species. Holarct Ecol. 2: 255-263.

Ekberg I, Eriksson G, Namkoong G, Nilsson C & Norell L (1994) Genetic correlations for growth rhythm and growth capacity at ages 3-8 years in provenance hybrids of *Picea abies*. Scand J For Res 9: 25-33.

Ekberg I, Eriksson G & Weng Y (1985) Between- and within-population variation in growth rhythm and plant height in four *Picea abies* populations. Stud For Suec 167: 1-14.

Emebiri LC, Devey ME, Matheson AC & Slee MU (1998) Age-related changes in the expression of QTLs for growth in radiata pine seedlings. Theor Appl Genet 97: 1053-1061.

Eriksson G, Andersson S, Eiche V, Ifver J & Persson A (1980) Severity index and transfer effects on survival and volume production of *Pinus sylvestris* in Northern Sweden. Stud For Suec 156: 1-31.

Eriksson G, Ekberg I, Dormling I & Matern B (1978) Inheritance of bud-set and bud-flushing in *Picea abies* (L.) Karst. Theor Appl Genet 52: 3-19.

Falconer DS & Mackay TFC (1996) Introduction to Quantitative Genetics. 4th ed. Longman Group Ltd, Harlow.

Fisher RA (1930) The genetical theory of natural selection. Oxford Univ. Press, Oxford.

Georges M, Nielsen D, Mackinnon M, Mishra A, Okimoto R, Pasquino AT, Sargeant LS, Sorensen A, Steele MR, Zhao X, Womack JE & Hoeschele I (1995) Mapping quantitative trait loci controlling milk production in dairy cattle by exploiting progeny testing. Genetics 139: 907-920.

Glavac D & Dean M (1993) Optimization of the single-strand conformation polymorphism (SSCP) technique for detection of point mutations. Hum Mutat 2: 404-414.

Hagner M (1970) A genecological investigation of the annual rhythm of *Pinus contorta* Dougl. and a comparison with *Pinus sylvestris* L. Stud For Suec 81: 1-23.

Haley CS, Knott SA & Elsen J-M (1994) Mapping quantitative trait loci in crosses between outbred lines using least squares. Genetics 136: 1195-1207.

Hayashi K & Yandell DW (1993) How sensitive is PCR-SSCP? Hum Mutat 2: 338-346.

Heide OM (1974) Growth and dormancy in Norway spruce ecotypes (*Picea abies*) I. Interaction of photoperiod and temperature. Physiol Plant 30: 1-12.

Henttonen H, Kanninen M, Nygren M, & Ojansuu R (1986) The maturation of *Pinus sylvestris* seeds in relation to temperature climate in northern Finland. Scand J For Res 1: 243-249.

Hyvärinen H (1987) History of forests in northern Europe since the last glaciation. Ann. Acad. Scient. Fenn. Series A III. Geologia-Geographica 145: 7-18.

Hänninen H, Häkkinen R, Hari P & Koski V (1990) Timing of growth cessation in relation to climatic adaptation of northern woody plants. Tree physiol 6: 29-39.

Jablanczy A (1971) Changes due to age in apical development in spruce and fir. Can For Serv Bimon Res Notes 27: 10

Jansen RC (1992) A general mixture model for mapping quantitative trait loci by using molecular markers. Theor Appl Genet 85: 252-260.

Jansen RC (1993) Interval mapping of multiple quantitative trait loci. Genetics 135: 205-211.

Jansen RC (1996) A General Monte Carlo method for mapping multiple quantitative trait loci. Genetics 142: 305-311.

Jansen RC, Johnson DL & Van Arendonk JAM (1998) A mixture model approach to the mapping of quantitative trait loci in complex populations with an application to multiple cattle families. Genetics 148: 391-399.

Jansen RC & Stam J (1994) High resolution of quantitative traits into multiple loci via interval mapping. Genetics 136: 1447-1455.

Jarvis SB, Taylor MA, MacLeod MR & Davies HV (1996) Cloning and characterization of the cDNA clones of three genes that are differentially expressed during dormancy-breakage in the seeds of Douglas Fir (*Pseudotsuga menziesii*). J Plant Physiol 147: 559-566.

Johnsen Ø & Apeland I (1988) Screening early autumn frost hardiness among progenies from Norway spruce seed orchards. Silva Fenn 22: 203-212.

Jonsson A, Eriksson G, Dormling I & Ifver J (1981) Studies on frost hardiness of *Pinus contorta* Dougl. seedlings grown in climate chambers. Stud For Suec 157: 1-47.

Karhu A, Hurme P, Karjalainen M, Karvonen P, Kärkkäinen K, Neale D & Savolainen O (1996) Do molecular markers reflect patterns of differentiation in adaptive traits of conifers? Theor Appl Genet 93: 215-221.

Kaya Z, Sewell MM & Neale DB (1999) Identification of quantitative trait loci influencing annual height- and diameter-increment growth in loblolly pine (*Pinus taeda* L.). Theor Appl Genet 98: 586-592.

Kearsey MJ & Hyne V (1994) QTL analysis: a simple 'marker-regression' approach. Theor Appl Genet 89: 698-702.

Kimura M (1983) The neutral theory of molecular evolution. Cambridge Univ. Press, Cambridge.

Knott SA, Elsen JM & Haley CS (1996) Methods for multiple-marker mapping of quantitative trait loci in half-sib populations. Theor Appl Genet 93: 71-80.

Knott SA, Neale DB, Sewell MM & Haley CS (1997) Multiple marker mapping of quantitative trait loci in an outbred pedigree of loblolly pine. Theor Appl Genet 94: 810-820.

Koski, V (1970) A study of pollen dispersal as a means of gene flow. Commun Inst For Fenn 70: 1-78.

Koski V & Sievänen R (1985) Timing of growth cessation in relation to the variations in the growing season. In: Tigerstedt PMA, Puttonen P & Koski V (eds) Crop physiology of forest trees. Helsinki University Press, Helsinki, Finland, p 167-193.

Koski V & Tallqvist R (1978) Tuloksia monivuotisista kukinnan ja siemensadon määrän mittauksista metsäpuilla (Results of long-time measurements of the quantity of flowering and seed crop of forest trees). Folia For 364: 1-60.

Kuittinen H, Sillanpää MJ & Savolainen O (1997) Genetic basis of adaptation: flowering time in *Arabidopsis thaliana*. Theor Appl Genet 95: 573-583.

Lai C, Lyman RF, Long AD, Langley CH & Mackay TFC (1994) Naturally occurring variation in bristle number and DNA polymorphisms at the *scabrous* locus of *Drosophila melanogaster*. Science 266: 1697-1702.

Lande R (1981) The minimum number of genes contributing to quantitative variation between and within populations. Genetics 99: 541-553.

Lande R & Thompson R (1990) Efficiency of marker assisted selection in the improvement of quantitative traits. Genetics 124: 743-756.

Lander E & Botstein D (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121: 185-199.

Lander E, Green P, Abrahamson J, Barlow A & Daley M, Lincoln S & Newburg L (1987) MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174-181.

Lanner RM (1976) Patterns of shoot development in *Pinus* and their relationship to growth potential. In: Cannell MGA & Last FT (eds) Tree physiology and yield improvement. Academic Press, New York, United States, p 223-243.

Li P & Adams WT (1993) Genetic control of bud phenology in pole-size trees and seedlings of coastal Douglas-fir. Can J For Res 23: 1043-1051.

Lin J-Z & Ritland K (1996) Construction of a genetic linkage map in the wild plant *Mimulus* using RAPD and isozyme markers. Genome 39: 63-70.

Luomajoki A (1993) Climatic adaptation of Scots pine (*Pinus sylvestris* L.) in Finland based on male flowering phenology. Acta For Fenn 237: 1-27.

Lynch M & Milligan BG (1994) Analysis of population genetic structure with RAPD markers. Mol Ecol 3: 91-99.

Lynch M & Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer Associates, Inc., Sunderland.

Mackay TFC (1995) The genetic basis of quantitative variation: numbers of sensory bristles of *Drosphila melanogaster* as a model system. Trends Genet 11: 464-470.

Mackay TFC & Langley CH (1990) Molecular and phenotypic variation in the *achaete-scute* region of *Drosophila melanogaster*. Nature 348: 64-66.

Maliepaard C & Van Ooijen JW (1994) QTL mapping in a full-sib family of an outcrossing species. In: Van Ooijen JW & Jansen J (eds) Biometrics in Plant Breeding: Applications of Molecular Markers, CPRO-DLO, Wageningen, p 140-146 .

Melchinger AE, Utz HF & Schön CC (1998) Quantitative trait locus (QTL) mapping using different testers and independent population samples in maize reveals low power of QTL detection and large bias in estimates of QTL effects. Genetics 149: 383-403.

Mikola J (1982) Bud-set phenology as an indicator of climatic adaptation of Scots pine in Finland. Silva Fenn 16: 178-184.

Mirov NT (1967) The genus Pinus. Ronald Press, New York.

Mitchell-Olds T (1996) Genetic constraints on life-history evolution: quantitative- trait loci influencing growth and flowering in *Arabidopsis thaliana*. Evolution 50: 140-145.

Mullis KB & Faloona FA (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Meth Enzymol 155: 335-350.

Muona O & Harju A (1989) Effective population sizes, genetic variability and mating system in natural stands and seed orchards of *Pinus sylvestris.* Silvae Genet 38: 221-228.

Neale DB & Williams CG (1991) Restriction fragment length polymorphism mapping in conifers and applications to forest genetics and tree improvement. Can J For Res 21: 545-554.

Orita M, Iwahana H, Kanazawa H, Hayashi K & Sekiya T (1989a) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci USA 86: 2766-2770.

Orita M, Suzuki Y, Sekiya T & Hayashi K (1989b) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5: 874-879.

Orr HA (1998) The population genetics of adaptation: the distribution of factors fixed during adaptive evolution. Evolution 52: 935-949.

Pan A, Hayes PM, Chen F, Chen THH, Blake T, Wright S, Karsai I & Bedö Z (1994) Genetic analysis of the components of winter hardiness in barley (*Hordeum vulgare* L.). Theor Appl Genet 89: 900-910.

Plomion C, Bahrman N, Durel C-E & O'Malley DM (1995) Genomic mapping in *Pinus pinaster* (Maritime pine) using RAPD and protein markers. Heredity 74: 661-668.

Plomion C, Durel C-E & O'Malley DM (1996) Genetic dissection of height in maritime pine seedlings raised under accelerated growth conditions. Theor Appl Genet 93: 849-858.

Powell W, Morgante M & Andre C, Hanafey M, Vogel J, Tingey S & Rafalski A (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breed 2: 225-238.

Prout T & Barker JSF (1993) F statistics in Drosophila buzzatii: Selection, population size and inbreeding. Genetics 134: 369-375.

Repo T, Zhang MIN, Ryyppö A, Vapaavuori E & Sutinen S (1994) Effects of freeze-thaw injury on parameters of distributed electrical circuits of stems and needles of Scots pine seedlings at different stages of acclimation. J Exp Bot 45: 823-833.

Rieseberg LH (1996) Homology among RAPD fragments in interspecific comparisons. Mol Ecol 5: 99-105.

Sarvas R (1972) Investigations on the annual cycle of development of forest trees: Active period. Commun Inst For Fenn 76: 1-110.

SAS (1987) SAS/STAT guide for personal computers, Version 6.1 Edition. Cary: NC: SAS Institute Inc.

Satagopan JM, Yandell BS, Newton MA & Osborn TC (1996) A Bayesian approach to detect quantitative trait loci using Markov Chain Monte Carlo. Genetics 144: 805-816.

Savolainen O & Hurme P (1997) Conifers from the cold. In: Bijlsma R & Loeschcke V (eds) Stress, Adaptation and Evolution, Birkhäuser Verlag, Basel, p 43-62.

Sax K (1923) The association of size differences with seed-coat pattern and pigmentation in *Phaseolus vulgaris.* Genetics 8: 522-560.

Sewell MM & Neale DB (1999) Mapping quantitative traits in forest trees. In: Jain SM & Minocha SC (eds) Molecular Biology of Woody Plants. Kluwer Academic Publishers, The Nederlands.

Sheffield VC, Beck JS, Kwitek AE, Sandstrom DW & Stone EM (1993) The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitution. Genomics 16: 325-332.

Sillanpää MJ & Arjas E (1998) Bayesian mapping of multiple quantitative trait loci from incomplete inbred line cross data. Genetics 148: 1373-1388.

Sillanpää MJ & Arjas E (1999) Bayesian mapping of multiple quantitative trait loci from incomplete outbred offspring data. Genetics 151: 1605-1619.

SkrØppa T (1982) Genetic variation in growth rhythm characteristics within and between natural populations of Norway spruce. A preliminary report. Silva Fenn 16: 160-167.

Tanksley SD (1993) Mapping polygenes. Annu Rev Genet 27: 205-233.

Thormann CE, Ferreira ME, Camargo LEA, Tivang JG & Osborn TC (1994) Comparison of RFLP and RAPD markers to estimating genetic relationships within and among cruciferous species. Theor Appl Genet 88: 973-980.

Toivonen A, Rikala R, Repo T & Smolander H (1991) Autumn colouration of first-year *Pinus sylvestris* seedlings during frost hardening. Scand J For Res 6: 31-39.

Uimari P & Hoeschele I (1997) Mapping linked quantitative trait loci using Bayesian analysis and Markov chain Monte Carlo algorithms. Genetics 146: 735-743.

Uimari P, Thaller G & Hoeschele I (1996) The use of multiple markers in a Bayesian method for mapping quantitative trait loci. Genetics 143: 1831-1842.

Ununger J, Ekberg I & Kang H (1988) Genetic control and age-related changes of juvenile growth characters in *Picea abies.* Scand J For Res 3: 55-66.

Vaartaja O (1959) Evidence of photoperiodic ecotypes in trees. Ecol Monogr 29: 91-111.

Weir BS (1996) Genetic data analysis II. Sinauer Associates, Inc., Sunderland.

Welsh J & McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18: 7213-7218.

Williams CG & Neale DB (1992) Conifer wood quality and marker-aided selection: a case study. Can J For Res 22: 1009-1017.

Williams JGK, Kubelik AR, Livak KJ & Rafalski JA (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18: 6531-6535.

Wu HG, Echt CS, Popp MP & Davis JM (1997) Molecular cloning, structure and expression of an elicitor-inducible chitinase gene from pine trees. Plant Mol Biol 33: 979-987.

Wu RL (1999) Mapping quantitative trait loci by genotyping haploid tissues. Genetics 152: 1741-1752.

Zeng Z-B (1993) Theoretical basis of separation of multiple linked gene effects on mapping quantitative trait loci with the aid of genetic markers. Proc. Natl. Acad. Sci. USA 90: 10972-10976.

Zeng Z-B (1994) Precision mapping of quantitative trait loci. Genetics 136: 1457-1468.

Original papers