GENETIC DETERMINISM OF AUTUMN FROST HARDINESS AND GROWTH PHENOLOGY DURING SEEDLING STAGE IN SCOTS PINE *(PINUS SYLVESTRIS L.)*

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Abstract:--Variation in first-year and second-year autumn frost hardiness, annual shoot elongation and growth phenology was studied within and between 18 fullsib families (appr. 100 individuals per family, of *Pinus sylvestris (L.),* representing north (=67 °N) x north, south (=62 °N) x north and south x south origins. Narrow sence heritablities, h^2, were $0.60< h^2<0.87$ for autumn frost hardiness, 0.66 and 0.58 for annual shoot elongation, 0.57 and 0.23 for growth termination in different growing seasons, and h2=0.25 for growth onset in the second year. One of the north x south families with 108 individuals was used for mapping quantitative trait loci for hardiness and growth traits by using 286 RAPD markers. 186 markers were found to fit into different linkage groups and 100 markers did not. 61 RAPD markers were found on 18 linkage groups for the female map with a total map distance of 601 cM, and 125 markers were found on 27 linkage groups for the male map with a total map distance of 1606 cM. The phenotypic variation explained by single or multiple markers on either the male or female map was up to 19.5% for frost hardiness, 23.7 % for growth termination and 30.8 % for annual shoot elongation. The proportion of additive variance explained by one or several QTLs on either the male or female map for years 1 and 2 was between 11.8 to 31.6% for frost hardiness, 41.5 to 52% for growth termination, and 18.6 to 46.6% for annual shoot elongation. Frost hardiness and shoot elongation were controlled by different QTLs on the male and female maps. QTLs for growth termination were only found on the female map. One QTL on the male map showed association to frost hardiness in both years. QTLs with pleiotropic effect on both frost hardiness and growth traits were not found in any linkage group. It is concluded that autumn frost hardiness and growth traits during seedling stage are under oligogenic control, with different QTLs located in different parts of the genome involved.

Keywords: *Pinus sylvestris,* Frost hardiness, Growth phenology, QTLs

INTRODUCTION

Survival of *Pinus sylvestris* in northern Sweden is clinally related to the latitude of origin (Eiche 1966; Remrod 1976; Eriksson et al.1980; Persson and Stahl 1990; Persson 1994). A phenological rhythm adapted to the short growing season is critical for the survival of forest trees in the north and an early cold acclimation in the autumn is probably the single most important factor for the survival of *Pinus sylvestris.* The cold acclimation process is initiated by increasing night length after midsummer, and since the last glacial period northern populations have adapted to initiate cold acclimation at shorter night length than more southerly populations (Nilsson and Eriksson 1986; Nilsson and Andersson 1987; Nilsson and Walfridsson 1993). Variation in cold acclimation rhythm between individuals, families and provenances can be studied by the artificial freezing test in late summer and autumn. Early cold acclimation is indicated by high cold resistance, and late cold acclimation by poor cold resistance in the freeze test. Transfer of stand seed from more northern localities and breeding for survival and volume production are of great economic importance for forestry in northern Sweden. Frost hardiness, growth capacity and growth phenology in *Pinus sylvestris* are complex characters and consist of several

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components of importance for adaptation. Autumn frost hardiness demonstrates a clinal variation in various populations of *Pinus sylvestris* from different latitudes throughout Sweden.

Molecular mapping of QTLs for frost hardiness and growth capacity can increase our knowledge of the distribution of genes throughout the genome involved in the expression of these characters. Information from molecular mapping can help to find DNA markers closely linked to important QTLs controlling frost hardiness and growth characteristics. Such markers can be used in future for early selection of breeding material (MAS).

MATERIALS AND METHODS

Experimental Material

Four north Swedish plus tree clones of *Pinus sylvestris* BD1027, BD1026, BD1238 and BD1217 originating from around latitude 67°N and four more southern plus tree clones Y2029, Y2023, Y2001 and Y2026 originating from around latitude 62°N were artificially crossed in a coastal clonal archive in northern Sweden (63°54'N) to obtain six full-sib families of each north x north, north x south, and south x south origins. The progeny test was sown in late October 1995 in 45 styrofoam containers filled with peat. The 18 families were randomly allocated to 40 single tree plots within every container. An average number of 100 seedlings per family (range 30-168) were raised (due to fungi attacks the average number was reduced to 87 in the second year) for testing growth and autumn cold hardening traits. The plants remained in the containers during the whole experimental period, including two full growth periods. One of the north x south families (BD1238 x Y2023) with 108 individuals was used for mapping QTL for the frost hardiness and growth characteristics.

Experimental Conditions

The first growth and cold acclimation periods were performed in greenhouse conditions with artificial control of photoperiod and temperature. Fertilization was made twice a week following a standard scheme and irrigation was supplied when necessary. After ten weeks of growth (4 h night length and 20/15 °C day night temperature), the seedlings were exposed to simulated autumn conditions by gradually increasing night length by 1 h week'" and reducing day/night temperatures to approximately 15/8 °C, to initiate cold hardening of the seedlings. During the second growth and cold acclimation periods the seedlings were exposed to the natural outdoor conditions in UmeA, Sweden (lat 63°50'N) from mid May until late September 1996 (after cold storage in darkness at –1°C between mid March and mid May).

Shoot Elongation Characteristics

The annual height growth was assessed at the end of the first and second growing periods. The growth rhythm of individual seedlings was characterized by the proportion of shoot elongation achieved on different occasions during the growing period relative the annual height growth. Onset of shoot elongation was defined as relative elongation on the occasion when the average value was close to 15 % (only assessed in the second year). Termination of shoot elongation was defined as relative elongation when the average value was close to 80 %. High values indicate early onset and cessation of shoot elongation.

Freeze Testing

The eighteen families were tested for variation in autumn frost hardiness by artificial freeze testing of detached needles from individual seedlings in early autumn at one year and two years of age. Freeze testing of detached needles allows the same individuals to be repeatedly tested on different occasions and temperatures. In this study, three (first year) or four (second year) needle samples were collected from each seedling for freeze testing at different temperatures. The needles were detached from the upper part of the seedlings and put in small moistened plastic bags immediately before (within 6 hours)

freeze testing. The limited number of needles allowed only small needle samples to be tested: first-yea] samples included one secondary needle pair and one primary needle, and second-year samples includec three needle pairs. Freeze testing was performed in programmable Weiss DC180PU freezing cabinets by gradual cooling of the air from 10 °C (cooling rate 8 °C 11 ˙') to predetermined test temperatures that were selected with regard to the actual hardiness levels of the test material. After 1.5 h at the selected test temperature the initial temperature was gradually resumed at a rate of 15 °C 11 ˙'. The large number of seedlings required freeze testing on three occasions during a one-week period, with one-third of the material tested on each occasion. After freeze testing the needles were removed from the plastic bags and stored for two weeks in high humidity environment (with needle bases in water) and similar temperature/photoperiod conditions as before freeze testing, until assessment of freezing injury. The injury was assessed by visual scoring of the proportion of discoloured needle tissue (10 %-classes) each test temperature. The freeze test procedure was mainly the same as used by Lindgren and Nilsson (1992) and Nilsson and Walfridsson (1993, 1995). Autumn frost hardiness of individual seedlings was calculated as both critical temperature and mean injury score at specific test temperatures. The critical temperature was estimated, using linear interpolation between adjacent test temperatures, as the temperature causing 50% discoloured needle tissue.

Random Amplified Polymorphic DNA (RAPD)

DNA was isolated from megagametophytes of germinating seeds and needle tissue by a CTAB procedure modified from Doyle and Doyle (1990). Proteinase K (1 mg/ml) was added before incubation at 60 °C. This method yields more than 1000 ng of DNA per megagametophyte. The minimum amount of DNA template used for RAPD PCR reaction was approximately 1 ng. RAPD PCR reactions were assembled in 96-well microtiter plates using different oligonucleotide primers purchased from Operon Technologies (Alameda, CA, USA). The polymerisation reaction was carried out by the Taq DNA polymerase from Promega. Samples were subjected to electrophoresis on a 1.5% (w / v) agarose gel in tris-borate EDTA buffer. The DNA fragments were separated after electrophoresis and stained with ethidium bromide.

The method for carrying out PCR to detect RAPD molecular markers follows Williams et al. (1990). RAPD PCR reaction volume was 13 µl in 96-well plates. Each well contained 1.3 ml 10 X PromegaTM PCR buffer, 1.06 ml of a 10 mM dNTP for each nucleotide, 2 ng template DNA in 3 ml of water, 15ng of a 10-mer from the collections of random primers (Operon Technologies, Alameda CA) suspended in 1.5 ml of water, 0.8 U of Taq polymerase and water to a total volume of 13 ml. PCR was carried out using an MJ Research 96-well thermocycler for 41 cycles consisting of denaturation for 1 minute at 92 °C, primer annealing for 1 minute at 37 °C, and primer extension for 2 minutes at 72 °C.

Six megagametophytes from the *Pinus sylvestris* clones BD1238 and Y2023 were screened with 10-mer primers to find candidate segregating RAPD markers. Segregating markers in parents were used to screen offspring. The north x south family BD1238 x Y2023, with 108 individuals, was used to detect QTL for frost hardiness and growth characteristics with 286 RAPD markers.

Quantitative Traits Measured

ELONG-Yl = Annual shoot elongation year 1 (mm)
ELONG-Y2 = Annual shoot elongation year 2 (mm)
TERM-Yl = growth termination year 1 (relative elongation, %, 10C weeks from sowing)
ONSET-Y2 = growth onset year 2 (relative elongation, %, on 7 June 1996)
TERM-Y2 = growth termination year 2 (relative elongation, %, on 4 July 1996)
CT-Y1 = Critical temperature (=autumn frost hardiness) year 1, all needles
CTPN-Y1 = Critical temperature (=autumn frost hardiness), primary needles, year 1
CT-Y2 = Critical temperature (=autumn frost hardiness) year 2

RESULTS AND DISCUSSION

Autumn Frost Hardiness

Autumn frost hardiness varied significantly ($p<0.001$) between and within family groups in both years (Table 1). The best frost hardiness was found for the north x north families, the poorest hardiness for the south x south families, and intermediate hardiness for the north x south families, which is in agreement with the well-known clinal adaptation of autumn cold acclimation to latitude of origin for *Pinus sylvestris* in Sweden. The phenotypic family-mean correlation of critical temperature between year 1 (cold acclimation under artificial conditions) and year 2 (cold acclimation under natural conditions) was high, r= 0.91. There was also a considerable overlap in frost hardiness between the three latitudinal origins, e.g. offspring from crosses between northern and southern clones have individuals with frost hardiness phenotypically corresponding both to the northern parental type and to the southern parental type. Narrow sense heritabilities for critical temperature were in the range $0.60<h^2<0.87$ and the additive genetic correlation between years was rA=0.88 (Table 1). Year-to- year correlations for critical temperature within the 18 families was, on average, r=0.43 (r=0.23 for the full-sib family used for QTL mapping) .

Table 1. Least square means in quantitative traits for north x north, north x south and south x south origins, and significance levels for the effect of geographical origin estimated from model 1 (growth traits) and model 2 (critical temperatures). Additive (VA) and nonadditive (VD) genetic variance components and narrow sense heritablities (h^2)were estimated by the program VDIAL based on all 18 families.

Trait	Parental origin			p-level, origin	VA	VD	h2
First year	north x north	north x south	south x south				
Shoot elongation, mm	100	99	87	<0.001	282	55	0.66
Growth termination, %	78	86	88	<0.001	0.0070	0.0024	0.57
Crit. temp. ° C	-11.7	-10.4	-8.6	<0.001	3.70	0.118	0.76
Crit. temp. primary needles ° C	-12.1	-10.8	-8.8	<0.001	3.52	0	0.60
Second year							
Shoot elongation, mm	115	136	147	<0.001	693	26	0.58
Growth onset, %	11.9	10.4	9.0	<0.001	0.0035	o.o000:	0.25
Growth termination, %	85.7	83.8	80.5	<0.001	0.0020	0.0004L	0.24
Crit. temp, ° C	-20.4	-17.9	-14.0	<0.001	14.65	0.648	0.87

Shoot Elongation

Significant ($p<0.001$) differences between and within family groups were found for all growth traits in both years (Table 1). In the first year, the family groups ranked from south x south to north x north for early growth cessation, and in the second year from north x north to south x south for both growth onset and growth termination (Table 1). The second year ranking is in accordance with the clinal trend of

earlier growth rhythm of northern compared with southern populations of Scots pine in Sweden, but the first year ranking is not. The difference might be involved through different genes in first year and second year growth rhythm, or in some kind of a short-term physiological after-effect that disappeared after the first growth period.

In a phytotron study with the same fullsib families of *Pinus sylvestris* produced on different localities, Dormling and Johnsen (1992) demonstrated that: (i) The parental environment affects growth and autumn frost hardiness in the first growth period, (ii) The effect on hardiness disappeared by the second growth period, (iii) The effect on growth was mainly mediated by the duration of the growth period, (iv) The effect of the parental environment could explain only a small part of the effects on growth and none of the effects on hardiness.

Because all seeds were produced on the same site and in the same year, any physiological after-effects from different large-scale maternal environments should have been eliminated. However, the mating program was based on just one or two trees per clone, growing in six plots (one plot per clone) distributed over abandoned farm land. Therefore, possible sources of physiological after-effects on first year seedling growth are, e.g. environmental differences between plots, different vitality of grafts affecting the seed development, and environmental differences between seed cones within tree crowns. Because seed size and germination characteristics were not measured, these theories cannot be confirmed. However, if physiological after-effects were present, the association between markers and quantitative traits should be underestimated in our study, at least in the first year. The narrow sense heritability (h^2) was 0.66 and 0.58 for first-year and second-year annual shoot elongation, respectively, 0.58 for cessation of first-year shoot elongation, and approximately 0.25 for both onset and cessation of second-year shoot elongation (Table 1).

Linkage Map Construction

In an earlier study a genetic map for *Pinus sylvestris* was constructed using RAPD markers segregated in 80 haploid megagametophytes of a single tree (Yazdani et al. 1995). 261 markers were mapped on 14 linkage groups with total map distance of 2638 cM and average distance between markers of 10.1 cM. Similar genome lengths for *Pinus* species are reported by Neale & Williams (1991) and Echt and Nelson (1997), suggesting similar rate of recombination among different pines.

The north x south family BD1238 x Y2023, with 108 F1 progeny, was used for mapping with RAPD markers. Of 286 RAPD markers all except 17 markers showed a simple mendelian segregation pattern with 1:1 or 1:3 proportion. We constructed linkage map for both parents based on informative markers segregating 1:1 and 3:1 in the F1 progeny. Of 286 markers, 186 were placed on different linkage groups and 100 markers did not fit any group (Figure 1). 61 markers were placed on 18 linkage groups on the female map with total map distance of 601 cM, and 125 markers on 27 linkage groups of the male map with total map distance of 1606 cM (Figure 2.) 25 markers appeared to be double heterozygotes in both parents. These intercross markers segregated 3:1 in the progenies and were used to join the male and female maps using JoinMap software. Six out of 25 intercross markers mapped on both male (M) and female (F) linkage group. These six markers which linked male and female linkage group together are as follows; marker J10/870 on linkage groups M1 and F16; markers F10/400, 002/700 and 002/1500 on linkage groups M2 and F13 and marker 004/320 on linkage groups M8 and F13. Linkage group F3 and M1 shared marker G04/800. Different linkage groups that share the same marker from double heterozygote parents correspond to homologs of the same chromosome. The map presented here is not a saturated map, therefore it needed more marker to be added in order to be able to decrease the number of the linkage groups to 12.

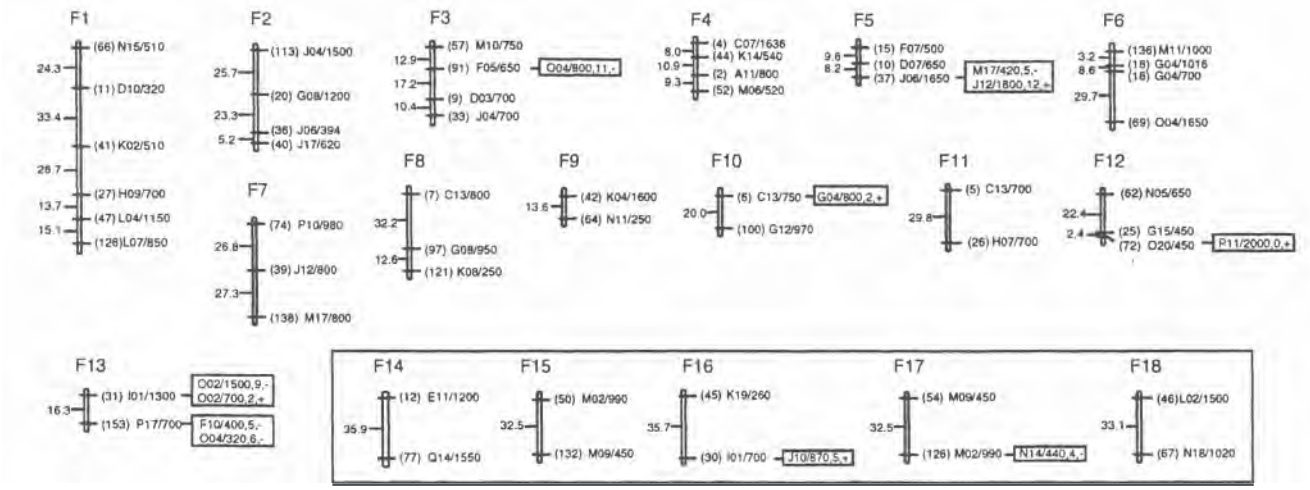

Figure 1. Female parental map (marker #1 to #78 one linkage phase "+", marker #79 to #156 the other linkage phase " – ". Squared markers segregate 3:1. Squared linkage groups were obtained with a LOD threshold >2.

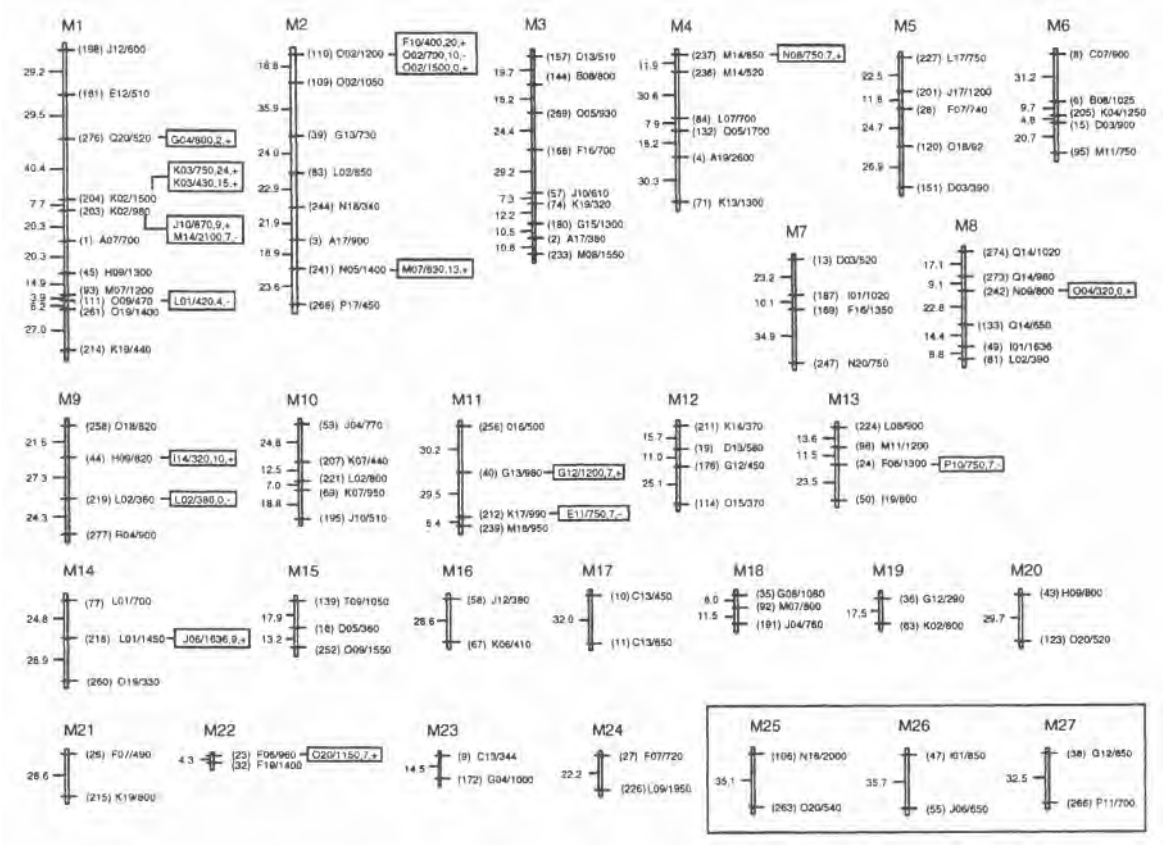

Figure 2. Male parental map. Marker #1 to #139 one linkage phase "+"; marker #140 to #278 the other linkage phase "-". Both maps were constructed with markers in the pseudo-testcross configuration (1:1). These markers were groups with a LOD threshold >3. Squared markers segregrate 3:1. Squared linkage groups were obtained with a LOD threshold >2.

Association Between Markers and Phenotypic Variation in Quantitative Traits

Most markers associated to quantitative traits were found on different linkage groups on the male and female maps, but a few markers associated to quantitative traits were not mapped on any linkage group (Table 2). On the male map, three markers D13/510, M14/850 and Q14/650 on linkage groups M3, M4, and M8 explained 23.8 % of the phenotypic variation in first-year shoot elongation and one marker K07/950 on linkage group M10 explained 10.8 % of the variation in second-year shoot elongation. On the female map, two markers K19/260 and L07/850 on linkage groups F16 and F1 explained 30.8 % of the phenotypic variation in shoot elongation in the first year, but no marker association was found for the second year. On the male map, no marker was associated with first-year growth cessation, but four markers G04/1016, L04/1150, and P17/700 on linkage group F16, Fl, F13 and M07/1050 on no linkage group on the female map accounted for 23.7 % of the phenotypic variation in the same trait. One unlinked marker 009/490 in the female map explained 12.1% of the phenotypic variation in second-year growth cessation. No marker on the male and female maps was associated with growth onset. Since no common marker was found explaining the variation for different growth components for years 1 and 2, it is presumed that annual height growth and growth rhythm (onset and termination of shoot elongation) in the seedling stage, are controlled by different QTLs. Maturation effect on QTL expression has already been reported (Plomion et al. 1996; Emebiri et al. 1998).

One marker J17/740 unlinked in the male map accounted for 15.2 % and one marker G15/450 linked to F12 on the female map accounted for 9.0 % of the phenotypic variation in first-year frost hardiness. Two markers J17/1200 and 101/850 on linkage group M5 and M26 on the male map accounted for 18.4% and two markers D03/700 and 101/700 on linkage group F3 and F16 on female map for 19.0 % of the variation in first-year frost hardiness of primary needles. One unlinked marker G13/740 in the female map explained 16.5 % of the variation in second-year frost hardiness.

The hardiness traits CTY1, FTY1 and CTPN-Y1, FTPN-Yl and CTY2, FTY2 *are essentially* the same, *only measured in different ways, therefore it is expected to find similar markers explaining the* phenotypic variation in these traits for frost hardiness. These markers are 101/850 on linkage group M26, J17/740 unlinked in the male map and G15/ 450 on linkage group F12 and G13/740 unlinked in the female map (Table 2).

For frost hardiness, as assessed by the mean injury score, the unlinked marker J17/740 showed association to various hardiness traits in year one and year two on the male map. The phenotypic variation for frost hardiness accounted for by the markers on the male and female maps as measured in different ways varied between 9.0 % and 19.5 % (Table 2).

Closely linked markers affecting first-year height and first-year growth termination were found on linkage group F1, and closely linked markers affecting first-year height and first-year frost hardiness of primary needles were found on linkage group F16.

Proportion of Additive Variance Explained By The QTLs

Proportions of the additive genetic variance explained by the QTLs, R^2G, indicate that genetic factors of large effect were involved for all investigated characters (Table 2). Single or multiple markers accounted for between 18.6% and 46.6% of the additive variation in annual shoot elongation, between 41.5% and 52% of the additive genetic variation in growth termination, and between 11.8% and 31.6% of the additive variation in autumn frost hardiness.

Table 2. QTL results for family BD1238 x Y2023. RAPD marker association with a QTL estimated by analysis of variance. Proportion of phenotypic variance (R^2_P) and proportion of additive genetic variance (R^2_G) explained by markers.

	Male map			Female map		
TRAIT	Marker (p-value, linkage group)	R^2_P	R^2_G	Marker (p-value,linkage group)	R^2p	R2
ELONG-Y 1	D13/510 (0.0035,M3) M14/850 (0.0065,M4) Q14/650 (0.0039,M8)	0.238	0.360	K 19/260 (0.0024, F16) L07/850 (0.001,F1)	0.308	0.466
ELONG-Y2	K07/950 (0.003,M10)	0.108	0.186			
TERM-Y 1				G04/1016 (0.0065,F6) L04/1150 (0.0092,F1) M07/1050 (0.0039,UL) P17/700 (0.0030,F13)	0.237	0.415
ONSET-Y2						
TERM-Y2				009/490 (0.0033,UL)	0.121	0.520
CTY1	J17/740 (0.0007,UL)	0.152	0.200	G15/450 (0.0035,F12)	0.090	0.118
CTPN-Y1	J17/1200 (0.0028,M5) 101/850 (0.0052, M26)	0.184	0.306	D03/700 (0.0074,F3) 101/700 (0.0082,F16)	0.190	0.316
CTY2				G13/740 (0.0002,UL)	0.165	0.189
FTY1	J17/740 (0.0021,UL)	0.129	0.169	G15/450 (0.0013,F12) M09/450 (0.0083,F17)	0.121	0.159
FTPN-Y1	101/850 (0.0022,M26)	0.117	0.195	N08/780 (0.006,UL)	0.108	0.180
FTY2	H09/820 (0.006,M9) J17/740 (0.0084,UL)	0.104	0.119	G13/740 (0.0002,UL) M17/800 (0.0043,F7)	0.195	0.224

UL=unlinked marker

Multiple R^2 is indicated when more than one marker was detected

CONCLUSION

We have found a number of QTLs for growth capacity and frost hardiness located either on different linkage groups or outside the map, but we did not find any common marker explaining the additive variation for both traits in seedling stage. This means that there is no QTL with pleiotropic effect on these two traits. However, we have two markers not so closely linked on linkage group F16, one showing association with annual height growth and the other with autumn frost hardiness in year 1. Development of autumn frost hardiness in *Pinus sylvestris* is assumed to be controlled by many loci with small effects and with additive gene action (Norell et al. 1986). With rapid development of DNA technology at present, it is now possible to test whether this assumption is correct or if there are major genes involved. Our result suggest that we are dealing with several regions controlling a major part of the variation for growth and frost hardiness, i.e., few genes with major effect and probably many genes with small effect are involved.

Major QTLs for frost response constitute a good opportunity for testing the candidate gene (CG) approach to characterize QTLs. Indeed, "functional" CG (i.e. known genes that putatively affect trait expression) could be mapped and their position compared with the localisation of the QTLs. " Positional " CG (i.e. genes that map within a QTL interval) could then be validated in different pedigrees.

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