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Quantitative genetics of spring and fall cold hardiness in seedlings from two Oregon populations of coastal Douglas-fir[☆]

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Abstract

Genetics of fall and spring cold hardiness were investigated in two western Oregon breeding populations (Coast and Cascade mountains) of Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco). Seedlings from 40 open-pollinated families from each population were grown in raised nursery beds and subjected to two soil-moisture regimes (well-watered and mild drought) to evaluate the influence of summer drought on ranking of families for cold hardiness. Artificial freeze testing (AFT) of detached shoots, followed by visual scoring of injury, was used to evaluate needle, stem and bud cold hardiness on three dates in the fall (September, October and November) after the second growing season, and once in the following spring (March).

The Cascade population suffered significantly less cold injury than the Coast population in fall AFT. However, in spring AFT the Cascade population was less cold hardy, although population differences were seldom significant. Families within both breeding zones varied significantly in cold hardiness, with mean estimates of individual heritabilities greater in spring ($\bar{h}_i^2 = 0.57$) than fall ($\bar{h}_i^2 = 0.37$), greater in the Coast ($\bar{h}_i^2 = 0.52$) than in the Cascade ($\bar{h}_i^2 = 0.42$) population, and greater in the wet ($\bar{h}_i^2 = 0.54$) than in the dry moisture regime ($\bar{h}_i^2 = 0.40$) (fall means based on October tests). A single test date seems adequate to assess fall cold hardiness, because estimated genetic correlations for cold injury between fall test dates were strong ($\bar{r}_A = 0.80$). Genetic correlations between spring and fall cold injury, however, were moderately negative ($\bar{r}_B = -0.66$ and -0.21 , Coast and Cascade, respectively), indicating that cold hardiness needs to be managed as two traits (i.e. fall and spring cold hardiness). Selection for cold hardiness based on a single shoot tissue is expected to increase cold hardiness in the other tissues as well, because genetic correlations between tissues in cold injury were moderately-to-strongly positive in both fall ($\bar{r}_B = 0.67$) and spring ($\bar{r}_B = 0.84$). Seedlings grown under summer drought incurred significantly less cold injury in the fall than those that were well-watered; nevertheless, strong genetic correlations in fall cold injury between moisture regimes ($\bar{r}_B = 0.91$) indicate that summer moisture conditions had little influence on family rankings for fall cold hardiness. Correlations of injury resulting from a natural frost event in November of the first year with injury from AFT in the fall of the second year ($\bar{r}_A = 0.72$ and 0.78 for needle and bud injury, respectively) confirmed that AFT reliably predicts cold hardiness to natural frost events. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Susceptibility to cold is among the most important factors limiting crop productivity and quality, and geographic distribution of temperate plant species (Sakai and Larcher, 1987; Chen et al., 1995). In forest trees, frost can result in injury, reduced growth, and death of seedlings (van Haverbeke, 1987; Timmis et al., 1994), saplings (van der Kamp and Worrall, 1990; Reich and van der Kamp, 1993; Balduman et al., 1999) and even mature individuals (Duffield, 1956). Modeling based on climate records and on empirical data for seedling hardening and dehardening, indicate that Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) in the Pacific Northwest is most susceptible to frost injury in October and the first half of November, and from mid-April to mid-May (Timmis et al., 1994). It is well established that cold hardiness varies among geographical sources of Douglas-fir in both the coastal variety (var. *menziesii*) (Campbell and Sorensen, 1973; Larsen, 1978; White, 1987; Loopstra and Adams, 1989; Schuch et al., 1989a, b) and interior variety (var. *glauca*) (Rehfeldt, 1979, 1986), as well as among families within geographical sources (Wheeler et al., 1990; White, 1987; Aitken and Adams, 1996, 1997).

Knowledge of the hardiness of genetic stock to fall and spring frosts is critical to the success of tree improvement programs, both for choosing which seed sources or families to plant in frost-prone sites, and for selecting and breeding for improved cold hardiness. In addition, selection for increased growth rate alone may indirectly result in unfavorable changes in cold hardiness (Rehfeldt, 1983; Aitken and Adams, 1995b), highlighting further the need for efficient cold hardiness screening methods and a better understanding of the genetic relationships between cold hardiness and other traits under selection.

Cold adaptation can be assessed by examining cold injury after natural frost events in field trials. There are, however, several limitations to relying on natural frost events for cold hardiness assessment. First, field tests are typically established only for the short term, on relatively mild, productive sites which seldom receive damaging frosts. Second, the effects of infrequent frosts may be confounded with injury due to other causes (e.g. disease, nutrient deficiency, waterlogging, drought). Third, the incidence, timing and

intensity of frosts are not generally uniform across test sites. Thus, statistical precision for testing cold hardiness differences among families or other genetic entities may be weak. One solution is to establish supplemental field tests on sites particularly susceptible to frost events, but this entails additional expense, and non-uniform freezing across the site may still be a problem. A better solution is to subject tissue samples to common test temperatures under controlled conditions in a freezer (i.e. artificial freeze testing, AFT) and, subsequently, evaluate the samples for cold injury (Burr et al., 1990). In this manner, objective and inexpensive estimates of cold hardiness can be obtained for large numbers of genotypes.

Previous studies by the Pacific Northwest Tree Improvement Research Cooperative (PNWTIRC) on the genetics of cold hardiness in coastal Douglas-fir employed saplings, due to their widespread and immediate availability in established progeny tests (Aitken and Adams, 1996, 1997; Balduman et al., 1999). Seedlings, however, are more prone to cold injury than saplings or older trees (van Haverbeke, 1987; Wheeler et al., 1990), because of their tendency to continue growing late in summer or early fall (Campbell and Sorensen, 1973; Rehfeldt, 1983; Li and Adams, 1993) and to burst bud before older trees (Irgens-Moller, 1967), their small size, and the proximity of their foliage to cold air pooling on the ground. In addition, testing for cold hardiness at the seedling stage has advantages over testing at older ages: seedling tests require less space and time than field tests and provide more uniform test conditions, resulting in greater statistical precision and cost effectiveness. Also, if ranking of families at the seedling stage is the same as in older trees, early testing for cold hardiness effectively evaluates cold hardiness at older ages as well.

The efficiency of seedling tests and ranking of families for different traits can be greatly influenced by the testing environment (Campbell and Sorensen, 1978; Kaya, 1992). In particular, summer soil-moisture availability has a strong effect on growth phenology and on cold hardiness of Douglas-fir seedlings (Joly et al., 1989). Seedlings from southwestern Oregon subjected to summer drought set bud earlier and were more frost hardy in early fall than those grown without drought stress (White, 1987). It is, therefore, important to determine the degree to which family

variation and ranking for cold hardiness within breeding populations is influenced by summer soil moisture conditions.

In this study, cold hardiness at the seedling stage was investigated using artificial freeze testing for the same two breeding populations, and the same forty families within each population, that were investigated earlier by the PNWTIRC in saplings (Aitken and Adams, 1996, 1997). Here, we evaluate the quantitative genetics of fall and spring cold hardiness in 2-year-old seedlings (Objective 1), as well as the influence of summer moisture availability on the inheritance of these traits, variation among families, and family rankings (Objective 2). In addition, a damaging natural frost in the fall following the first growing season provided an opportunity to evaluate the degree to which artificial freeze testing predicts susceptibility to injury from natural frost events (Objective 3). The relationship between cold hardiness at the seedling and sapling stages is addressed in another paper (O'Neill et al., 2000).

2. Materials and methods

2.1. Materials

The open-pollinated Douglas-fir seed used in this study came from 40 phenotypically selected parent trees (i.e. 40 families) within each of two western Oregon breeding zones (populations) — a Coast breeding zone (US Forest Service Region 6 Breeding Unit 12021) located in the Siuslaw National Forest in the Coast Range and centered at latitude 44°20'N, longitude 123°50'W, and a Cascade breeding zone (Bureau of Land Management Breeding Unit 33), located on the lower west slope of the Cascade Mountains and centered at latitude 44°50'N, longitude 122°30'W. Parent trees in the Coast breeding zone came from an area of $\approx 500 \text{ km}^2$, and an elevational range of 67 to 333 m. In the Cascade breeding zone, parents came from a smaller area ($\approx 150 \text{ km}^2$), but from a larger elevational range (300–833 m).

2.2. Experimental design

Seedlings were grown in raised nursery beds (1.3 m \times 16 m \times 0.7 m deep) in Corvallis, OR. The

experimental design was a split-plot with four randomized complete blocks. The two main plots (moisture regimes) in each block were subdivided into two replicate sub-blocks (A and B), each containing all of the 80 families, randomly allocated to sub-plots. Two replicate sub-blocks of the families were needed in each main plot to ensure that adequate numbers of shoots were available for artificial freeze testing. Each family sub-plot consisted of a four-tree row of seedlings. Thus, there was a total of 4 seedlings/sub-plot \times 80 sub-plots/sub-block \times 2 sub-blocks/main plot \times 2 main plots/block \times 4 blocks = 5120 test seedlings. Extra seedlings from the test families were used as buffers, with one buffer row around each main plot, and three buffer rows between sub-blocks.

2.3. Seedling culture

Each raised bed was divided into 4-m long main plots, with plastic sheets lining the inside walls of the plots to prevent water movement between them, and landscape cloth laid under the beds to prevent seedling roots from penetrating the ground to access water. The beds were filled with sterilized loamy-sand, and peat was mixed into the top 20 cm at a 1:1 ratio.

Stratified seed were sown at a spacing of 8 cm within a row and 10 cm between rows in early April 1995. Three seed were sown per sowing hole, and thinned at random to a single seedling in the summer. Seedlings were irrigated and fertilized as needed in the first growing season to promote growth (O'Neill, 1999).

Two moisture regimes, well-watered (wet) and mild water stress (dry), were initiated early in the second growing season (mid-June of 1996). A clear plastic canopy was erected over the beds during rainy periods. Soil water potentials were maintained at roughly -0.8 and -1.7 MPa in the wet and dry regimes, respectively, by irrigating to field capacity (wet regime) or with ≈ 1.0 cm of water (dry regime) when pressure chamber estimates of average pre-dawn soil water potentials of buffer seedlings fell below -1.0 (wet regime) or -2.0 MPa (dry regime). Moisture treatments ended on 18 October 1996, when plots of both regimes were watered to field capacity and further exclusion of natural precipitation was stopped. Seedling survival at the end of the experiment was 98%.

2.4. Artificial freeze tests

Cold injury from artificial freeze tests (AFTs) was assessed on three occasions after bud set in the second growing season (i.e. in September, October and November 1996) and once prior to budburst in the third growing season. AFTs were applied on multiple dates in the fall because earlier work with saplings showed that family ranking for cold hardiness are less stable across dates in the fall than in the spring (Aitken and Adams, 1996, 1997). We wished to determine whether fall sampling date is an important factor for ranking cold hardiness at the seedling stage.

On each AFT date, the terminal 5 cm of two lateral shoots was harvested from each sampled seedling and subjected to freezing; one shoot at each of two test temperatures (selected based on preliminary test results - see below), followed by visual evaluation of tissues for cold injury. Two shoot samples were harvested from each seedling in sub-block 'A' in the September and October tests (begun 20 September and 12 October) and from each seedling in sub-block 'B' in the November and March tests (begun 16 November and 24 March). Second-order branch tips were selected from the middle third of each seedling to minimize sampling within-seedling variation in cold hardiness. No attempt was made to avoid second flushed branch tips which constituted about 5% of the samples. On each test occasion, shoots were processed by block, with all seedlings from a single block comprising the sample of a single day. Sampling and freezing of all four blocks required eight days (2 days per test temperature per block) on each test occasion.

Upon harvesting, each shoot was labeled and placed in an ice chest, then transferred to a refrigerator (2°C) until samples were packaged for the freeze tests. Groups of ≈ 50 shoots were wrapped into flat packets, first in cheesecloth moistened with tap water, and then in aluminum foil. Ends and sides of the packets were pinched closed to minimize desiccation. The packets were placed on a thick aluminum shelf (to facilitate cooling through conduction rather than convection) in a freezer containing a temperature controller, and held a minimum of 7 h at -2°C, in order to freeze extracellular water. The temperature was then lowered to the test temperature at a rate of 3–5°C/h (Glerum, 1985) and maintained at the test temperature for 1 h,

whereupon the packets were removed from the freezer and placed in a refrigerator (2°C) to thaw slowly. Once thawed, the packets were transferred to laboratory benches (16–18°C) where they were held for 6–8 days in order for signs of cold injury to develop in the dark, humid and warm environment inside the aluminum foil packets (Burr et al., 1990).

As needles, stems and buds may differ in genetic control and variation for cold hardiness (Aitken and Adams, 1996) as well as in their ability to predict cold hardiness in the field (Simpson, 1983), all three shoot tissues were scored visually for injury. Stems and terminal buds were split lengthwise to reveal damage (i.e. browning and yellowing of normally green tissues). Needle damage was evidenced by graying, browning or abscission of needles. Injury to each tissue was recorded as the percentage (to the nearest 10%) of tissue damaged. All samples from one block were scored by a single individual to reduce experimental error.

Discrimination among families for cold hardiness is best achieved when family variance in cold injury is the greatest, which usually occurs at test temperatures causing an intermediate level of mean injury (i.e. 30–70%) (Aitken and Adams, 1996). Consequently, two test temperatures (T1 and T2) were employed on each of the four AFT test dates, to better ensure that the target of 30–70% mean injury would be obtained. On each AFT date, two test temperatures, applied to all seedlings regardless of source population or moisture regime, were selected on the basis of preliminary AFTs performed 1 week prior on shoots from buffer seedlings (O'Neill, 1999). Test temperatures of -8 and -12°C (September), -12.5 and -15.5°C (October), -20 and -22°C (November), and -15 and -19°C (March) were selected through interpolation from preliminary test injury scores.

2.5. Natural frost injury

Delayed onset of dormancy during the first fall, evidenced by second-flushing of apical or lateral buds of $\approx 20\%$ of the seedlings, may have been brought about, in part, by the Lygus bug (*Lygus hesperidus* Hahn) injury (4% of the seedlings were injured by the insects) or fall fertilization, and may have predisposed the seedlings to cold injury from freezes during the early mornings of 1–5 November 1995. Minimum

daily temperatures recorded at 1 m above ground level by the Environmental Protection Agency Corvallis laboratory, located 700 m from the nursery, and within a few meters elevation, averaged -4.5°C (-3.7 to -5.0°C) over this 5-day period.

Cold injury resulting from the early November frosts made it possible to compare family cold hardiness in a natural frost (NF) to that determined by AFT. Injury from the natural frosts was evidenced by rust colored foliage 1 week after the frosts, and by aborted apical buds the following spring. Damage was more severe to the apical shoot and the tips of the upper branches than to branches at the bottom of the tree. The most severely injured seedlings (11% of total) developed a stunted, 'bushy' appearance following the NF event, which persisted through the third growing season, but none of the seedlings were killed by the frost. NF cold injury was recorded on each seedling by visually estimating the percent of foliage injured (FI), to the nearest 10%, on 12 November 1995, and by noting apical bud mortality (ABM) in the spring after the frost.

Only non-damaged lateral shoots were used for the AFTs after the second growing season (i.e. 1996). The NF in fall, 1995, is not expected to have affected cold hardiness of non-damaged shoots the following year (Les Fuchigami, Department of Horticulture, Oregon State University, personal communication). Three observations support this opinion. First, all seedlings, including those which were heavily damaged, produced healthy foliage on non-damaged shoots in the spring of 1996. Second, buds which developed on damaged seedlings in the fall of 1996 were large and healthy, and formed within the usual bud set period. Third, seedlings did not appear to sustain any NF injury during the winter of 1996–1997.

2.6. Statistical analysis

Cold hardiness was evaluated using 12 AFT traits (i.e. cold injury scores of three tissues \times four test dates). Preliminary analyses examined family variation in AFT injury at the two test temperatures (T1 and T2) separately, and the mean injury score (T12) averaged across both temperatures (T1 and T2). In 47 of 48 cases (12 AFTAFT traits \times 2 populations \times 2 moisture regimes), family variance of mean injury scores (T12) was greater than when scores from either

of the temperatures were used individually. Consequently, all AFT analyses were based on mean injury scores across two temperatures.

The 12 AFT traits were subjected to three analyses of variance. To test the effects of moisture treatments and moisture-by-family interaction, data for each population were analyzed separately across both treatments (Analysis 1). To assess the relative effectiveness of each moisture regime for detecting family differences, data for each population were analyzed separately for each moisture treatment (Analysis 2). To assess differences between the two populations, data for the populations were analyzed together, but separately for each moisture regime (Analysis 3).

Analysis 1 was conducted using the following linear model for a split-plot design:

$$Y_{ijkl} = \mu + M_k + B_j + MB_{jk} + F_i + MF_{ik} + FMB_{ijk} + e_{ijkl} \quad (1)$$

where Y_{ijkl} is the individual seedling injury score, μ the overall population mean, M_k the effect of the k th moisture regime, B_j the effect of the j th block, MB_{jk} the main plot error, i.e. the interaction of moisture regime and block, F_i the effect of the i th family, MF_{ik} the interaction of moisture regime with family, FMB_{ijk} the interaction of family with moisture regime and block, e_{ijkl} the within-family plot error.

All effects, except moisture regime, were considered random. Sub-block is not a factor in any of the analyses of AFT traits because seedlings from only one sub-block were sampled on each AFT occasion. Analysis 2 used the same model as Analysis 1, with all factors containing moisture regime removed. Analysis 3 used the same model as Analysis 2, with the addition of factors for population (fixed effect) and population-by-block effects, and with families nested within populations. Natural frost injury traits (FI and ABM) were analyzed with the model used in Analysis 2 (i.e. separately by population, moisture treatment and sub-block; 16 seedlings per family) in order to facilitate comparison of genetic parameters (heritabilities, family coefficients of variation, and genetic correlations with AFT traits) between natural frost injury traits and AFT traits. Because FI and ABM were measured on all seedlings and before the imposition of the drought, genetic parameters for FI and ABM were averaged over the two sub-blocks and moisture treatments.

For the purposes of testing moisture treatment, population, family, and family-by-moisture treatment interaction effects, Type III sums of squares were calculated for all traits in the above analyses using the SAS GLM procedure (SAS, 1996). In all cases but two, *F*-tests were straightforward (Montgomery, 1991, Sections 7.4 and 14.2). To test moisture regime (Analysis 2) and population effects (Analysis 3), approximate (i.e. 'pseudo') *F*-tests were necessary (Montgomery, 1991, Section 8.3).

Examination of residuals for the AFT traits indicated that, with few minor exceptions, errors were distributed normally and with homogenous variance across treatments. Thus, significance tests of all AFT traits were performed on non-transformed data. Significance tests were performed on log-transformed values of FI, and on log-transformed plot-mean values of ABM.

All variance component estimates required for genetic parameter estimates were derived using the restricted maximum likelihood (REML) estimator in the MIXED procedure of SAS (SAS, 1996). To assess the strength of genetic control of AFT and NF cold injury traits, individual heritabilities were calculated as:

$$h_i^2 = \frac{3\sigma_F^2}{\sigma_F^2 + \sigma_{FB}^2 + \sigma_e^2} \quad (2)$$

The additive genetic variation (numerator in the heritability equation) was estimated as three times the family variance, rather than four times (as appropriate for half-sib families), because open-pollinated Douglas-fir progeny are expected to be more closely related than true half-sibs (Squillace, 1974; Campbell, 1979). Individual heritabilities of the binary trait, ABM, were converted to an underlying scale in order to free them of the effect of mean incidence (Lynch and Walsh, 1998).

Standard errors of individual heritability estimates were estimated according to Dickerson (Dickerson, 1969, pp. 49–50), using the asymptotic variances of variance components derived in the SAS VARCOMP procedure.

Genetic relationships (i.e. genetic correlations) between AFT cold injury scores in different test periods, between tissues in a single test period, and between natural and artificial frost injury for paired tissues (i.e. October AFT needle injury and FI; and

October AFT bud injury and ABM), were evaluated separately by moisture regime. Also evaluated were genetic relationships between the same AFT cold injury trait measured in the dry and wet regimes. Type A genetic correlations (r_A) were estimated for traits measured on the same seedlings (i.e. when both traits were measured in the same sub-block) (Falconer, 1986, p. 284):

$$r_A = \frac{\text{Cov}_{F_{xy}}}{\sqrt{\sigma_{F_x}^2 \times \sigma_{F_y}^2}}, \quad (3)$$

where $\text{Cov}_{F_{xy}}$ is the estimated family covariance between traits x and y , and $\sigma_{F_x}^2$ and $\sigma_{F_y}^2$ the estimated family variances of traits x and y , respectively. $\text{Cov}_{F_{xy}}$ was calculated from:

$$\text{Cov}_{F_{xy}} = \frac{(\sigma_{F_{x+y}}^2 - \sigma_{F_x}^2 - \sigma_{F_y}^2)}{2} \quad (4)$$

where $\sigma_{F_{x+y}}^2$ is the estimated family variance of $(x + y)$.

Type B genetic correlations (r_B) were calculated between traits measured on different seedlings (i.e. when the two traits were measured on different main plots (moisture regimes) or different sub-blocks within the same main plot) (Burdon, 1977):

$$r_B = \frac{\text{Cov}_{\bar{F}_{xy}}}{\sqrt{\sigma_{F_x}^2 \times \sigma_{F_y}^2}} \quad (5)$$

where $\text{Cov}_{\bar{F}_{xy}}$ is the covariance of family mean scores for traits x and y .

Each AFT trait was assessed in only one sub-block, whereas both NF cold injury traits were scored on all seedlings, therefore genetic correlation estimates between AFT and NF cold injury traits were calculated in two ways: as type A genetic correlations, using data for the same sub-block for both traits, and as Type B genetic correlations, using data from different sub-blocks for the two traits. The two genetic correlation estimates (r_A and r_B) for each trait pair were averaged to improve the precision of the estimates.

3. Results

3.1. Genetic variation in AFT scores

For each tissue, family rankings for cold hardiness were similar for the three fall test dates (mean

Table 1

Estimated population means, ranges in family means, family coefficients of variation (CV_F (%)), and individual heritabilities (h_i^2) for artificial freeze testing (AFT) scores (percentage tissue damage) of seedlings within two Oregon breeding zone populations of Douglas-fir (Coast and Cascade) grown in dry and wet summer moisture environments

Test date ^a	Tissue	Coast				Cascade				F_{pop}^c
		Population mean	Range of family means	CV_F (%) ^{b,c}	$(h_i^2)^d$	Population mean	Range of family means	CV_F (%) ^{b,c}	$(h_i^2)^d$	
<i>Dry</i>										
Oct	Needle	47.3	20.7–74.0	20.4	0.31	30.2	11.8–54.2	25.8	0.24	***
	Stem	43.8	18.1–68.1	28.2	0.53	24.7	6.9–47.9	28.4	0.29	***
	Bud	57.3	29.9–76.8	16.9	0.33	37.9	14.2–63.9	25.6	0.35	***
	Mean	49.5		21.8	0.39	31.0		26.6	0.29	
Mar	Needle	61.9	34.4–88.6	15.0	0.46	63.2	30.5–81.6	15.4	0.50	ns
	Stem	62.7	43.4–82.3	12.6	0.47	64.0	47.9–84.7	11.7	0.44	ns
	Bud	62.6	46.9–86.1	13.0	0.52	63.4	49.7–83.7	11.6	0.41	ns
	Mean	62.4		13.5	0.48	63.5		12.9	0.45	
<i>Wet</i>										
Oct	Needle	81.6	66.0–96.5	6.6	0.19	71.4	41.7–90.4	11.5	0.32	*
	Stem	69.4	33.7–97.2	20.3	0.66	50.5	23.3–84.0	22.0	0.35	***
	Bud	62.6	27.4–88.3	21.5	0.47	43.1	10.8–78.2	28.9	0.43	***
	Mean	71.2		16.1	0.44	55.0		20.8	0.37	
Mar	Needle	58.6	31.9–85.1	19.6	0.67	66.2	40.3–91.5	14.4	0.52	*
	Stem	58.2	40.4–88.1	19.3	0.83	64.5	48.3–86.8	13.5	0.55	*
	Bud	60.8	44.1–87.4	16.8	0.78	65.1	47.6–87.8	13.8	0.70	ns
	Mean	59.2		18.6	0.76	65.2		13.9	0.59	

^a October tests conducted in 1996; March tests conducted in 1997.

^b CV_F (%) = $100 \times (\text{square root of family variance})/\text{population mean}$.

^c Family variance component significant at $P < 0.001$ in all cases.

^d Estimated standard errors of h_i^2 averaged 0.14 (range 0.07–0.23).

^e Significance of difference between population (Coast vs. Cascade) means: ns, not significant; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

$r_A = 0.80$). The only exception involved September–November and October–November correlations for buds, as buds suffered severe injury in AFTs in November. The strongest heritabilities among the three fall test dates were observed in October. Therefore, only October AFT test results are presented for fall cold hardiness, and 'fall' cold hardiness is used synonymously with 'October' cold hardiness in the remainder of this article.

Within a population, moisture regime and test date, mean damage to the three tissues was fairly similar (Table 1). Cold injury following AFT was significantly greater ($P \leq 0.05$) in the Coast (60.3%) than in the Cascade (43.0%) population for all tissues in the fall. In contrast, AFT injury scores were higher for the Cascade than for the Coast population in spring, but differences were small and seldom significant.

Significant family differences in AFT scores were found for all combinations of test date, tissue type, moisture regime and population, with ranges among family means often large (Table 1). For example, the hardiest family in the Coast population, when grown in the wet regime, suffered only 34% stem injury in October, while the least hardy family suffered 97% injury. The ability to detect family differences in cold injury was facilitated by the attainment, in most cases, of intermediate population mean injury scores (i.e. 30–70%).

Estimates of individual heritabilities of AFT scores were generally low-to-moderate, but varied considerably over seasons and breeding zones (Table 1). On average across tissues and moisture regimes, heritabilities were greater in spring ($\bar{h}_i^2 = 0.57$) than fall ($\bar{h}_i^2 = 0.37$), and somewhat greater in the Coast

breeding zone ($\bar{h}_i^2 = 0.52$) than in the Cascade zone ($\bar{h}_i^2 = 0.42$). Averaged across populations, moisture regimes and test dates, heritabilities were comparable for stems ($\bar{h}_i^2 = 0.51$) and buds ($\bar{h}_i^2 = 0.50$), but slightly lower for needles ($\bar{h}_i^2 = 0.40$). Heritabilities were weak when mean injury scores were <30% or >70%.

3.2. Influence of moisture regime on cold hardiness

As expected, drought accelerated fall hardening; in all cases in October, mean AFT scores were less in seedlings grown in the dry regime (Table 1), although differences were not significant for buds (Table 2). The effect of summer drought on cold hardiness dissipated by spring, as differences in AFT scores between moisture regimes were small and non-significant in March (Tables 1 and 2).

Heritability estimates were lower in the dry than in the wet regime, in both October ($\bar{h}_i^2 = 0.34$ — dry; $\bar{h}_i^2 = 0.40$ — wet) and March ($\bar{h}_i^2 = 0.47$ — dry; $\bar{h}_i^2 = 0.67$ — wet) (Table 1), although family variation for both traits was similar in the two moisture environments. Moisture regime also appeared to have little influence on the ranking of families for cold hardiness. Type B genetic correlations between cold injury scores in the dry and wet regimes were strong ($\bar{r}_B = 0.87$), and moisture regime-by-family interaction was significant only for needles and stems in spring (Table 2).

Table 2

Significance of moisture regime (dry vs. wet) (M) and moisture regime-by-family (M × F) effects, and estimated Type B genetic correlations (r_B) between moisture regimes for artificial freeze testing (AFT) scores (percentage of tissue damage)

Test date	Tissue	Breeding zone					
		Coast			Cascade		
		M ^a	M×F ^a	r_B	M ^a	M×F ^a	r_B
Oct	Needle	*		1.00 ^b	**		0.81
	Stem	**		0.93	**		0.89
	Bud			1.00 ^b			0.81
	Mean			0.98			0.84
Mar	Needle		*	0.83	**		0.62
	Stem		*	0.86	*		0.84
	Bud			1.00 ^b			0.87
	Mean			0.89			0.78

^a Only cases where these effects were significant (*, $P < 0.05$; **, $P < 0.01$) are noted.

^b Genetic correlation estimate exceeded 1.00.

Table 3

Estimated genetic correlations (r_A) of artificial freeze test (AFT) scores between needles, stems and buds in October 1996 and March 1997

Test date	Correlation ^a	Breeding zone	
		Coast	Cascade
Oct	Needle-stem	0.97	0.64
	Needle-bud	0.54	0.36
	Stem-bud	0.78	0.73
Mar	Needle-stem	0.76	0.89
	Needle-bud	0.62	0.82
	Stem-bud	0.95	0.96

^a Correlations averaged over dry and wet moisture regimes.

3.3. Genetic correlations between tissues and between sampling dates

Estimated genetic correlations between AFT scores of different tissue types were strong in March ($\bar{r}_A = 0.84$) and moderately strong in the fall ($\bar{r}_A = 0.67$) (Table 3). Correlations involving stems (i.e. stem-needle and stem-bud correlations) were slightly stronger, on average ($\bar{r}_A = 0.84$) than correlations involving needles (i.e. needle-stem and needle-bud correlations) ($\bar{r}_A = 0.70$) or buds (i.e. bud-needle and bud-stem correlations) ($\bar{r}_A = 0.72$).

Genetic correlations for injury scores between fall and spring sampling dates were moderately strong and negative in the Coast population ($\bar{r}_B = -0.66$) and

Table 4

Estimated type B genetic correlations^a (r_B) for artificial freeze test (AFT) scores between fall (October) 1996 and spring (March) 1997 test dates

Tissue	Breeding zone	
	Coast	Cascade
Needle	−0.75	−0.21
Stem	−0.47	−0.22
Bud	−0.74	−0.20
Mean	−0.66	−0.21

^a Correlation estimates were averaged over dry and wet moisture regimes.

weakly negative in the Cascade population ($\bar{r}_B = -0.21$) (Table 4).

3.4. Natural frost injury

Only about 3% of the foliage, on average, was injured by the natural freeze event in November 1995, while the apical bud of approximately 30% of the seedlings was killed (Table 5). Estimated individual heritabilities were larger for apical bud mortality (ABM) ($\bar{h}_i^2 = 0.33$) than for foliage injury (FI) ($\bar{h}_i^2 = 0.22$), but similar to that for fall AFT scores ($\bar{h}_i^2 = 0.37$) (Tables 1 and 5). Estimated genetic correlations were moderately strong between FI and October AFT needle scores ($r_B = 0.72$), and between ABM and October AFT bud scores ($r_B = 0.78$).

Table 5

Estimated population means, ranges in family means, family coefficients of variation (CV_F (%)), and individual (h_i^2) heritabilities for two measures of injury (FI and ABM) to 1-year-old seedlings from two Oregon breeding zone populations (Coast and Cascade) after a natural frost event in November 1995^a

	FI		ABM	
	Coast	Cascade	Coast	Cascade
Population mean (%)	3.7	2.2	36	21
Family range	0.3–9.4	0.3–9.8	11–67	6–44
CV_F (%) ^b	50.3	69.2	35.2	37.5
$(h_i^2)^c$	0.23	0.21	0.40	0.26
r_A^d	0.94	0.50	0.88	0.67

^a FI, foliage injury— visual estimate of the percent of foliage injured; ABM, apical bud mortality — percent of trees with a frost-killed apical bud. Also shown are estimates of genetic correlations between (a) FI and artificial freeze test (AFT) injury scores to needles, and (b) ABM and AFT injury scores to buds, for AFTs conducted the following fall (October 1996). All genetic parameters are averages for the four moisture treatment × sub-block combinations.

^b Family variance significant in all cases at $P < 0.001$.

^c Estimated standard errors for $h_i^2 = 0.09 - 0.14$.

^d Genetic correlations are averages over type A and B genetic correlations (see text).

4. Discussion

4.1. Influence of environmental factors on cold hardiness

Cold hardening is usually triggered by photoperiod and then by decreasing fall temperatures (Weiser, 1970; Rehfeldt, 1979, 1980; Nilsson and Walfridsson, 1995). However, it can be hastened by several factors including chilling (Weiser, 1970), soil moisture (Timmis and Tanaka, 1976; White, 1987; Balduman et al., 1999), nutrition (Weiser, 1970; Alden and Hermann, 1971), and light intensity (van den Driessche, 1970), whereas *dehardening* is primarily heat-sum dependent after chilling requirements have been met (Campbell and Sugano, 1975; Thomson and Moncrieff, 1982). In agreement with earlier studies (Timmis and Tanaka, 1976; White, 1987), the mild drought stress hastened hardening, so that seedlings in the dry treatment experienced less cold injury after AFT, even into November, one month after the drought treatment was terminated. By spring, however, the effects of drought on mean cold hardiness had disappeared (Tables 1 and 2).

4.2. Genetic variation and control of cold hardiness

Somewhat weak genetic control of AFT injury in the fall, moderate control of AFT injury in spring, but strong family variation for these traits in both seasons

(Table 1) corroborate results of studies of cold hardiness of sapling-age trees in the same families (Aitken and Adams, 1996, 1997; O'Neill et al., 2000), and confirm the amenability of these traits to genetic improvement. However, temperature at the time of female flowering and embryogenesis can affect cold hardiness of the ensuing progeny (Johnsen et al., 1996). Consequently, temperature differences among parent tree environments in the months prior to seed collection may have upwardly biased estimates of genetic variation for cold hardiness traits.

Weaker estimated heritabilities of AFT traits in fall ($\bar{h}_i^2 = 0.37$) than in spring ($\bar{h}_i^2 = 0.57$) may be due to the greater number of factors influencing acclimation in the fall, i.e. the greater number of environmental cues in fall may increase environmental variation influencing the expression of hardiness. Also, because spring cold hardiness is primarily heat sum dependent, fewer genes may regulate spring cold hardiness and less environmental 'noise' may influence the trait.

Differences in AFT cold injury between populations, in all cases in fall, and in some cases in spring, reflect adaptation to different source environments. Greater cold hardiness in the Cascade population in fall is likely a response to more rapidly decreasing minimum daily temperatures during fall in the Cascade region, which is further from the ocean and slightly higher in elevation than the Coast population. Similarly, more rapidly increasing minimum daily temperatures during spring in the Cascade region likely resulted in adaptation for lower heat-sum requirements for budburst and dehardening for the Cascade population (Campbell and Sugano, 1979; Rehfeldt, 1979; Steiner, 1979; Balduman et al., 1999). Consequently, Cascade seedlings dehardened more quickly and were less hardy in spring than Coastal seedlings (but significantly so only in the wet treatment).

Heritabilities for fall and spring cold hardiness were slightly stronger in the Coast than in the Cascade population. This tendency was also observed with the same families at the sapling stage (Aitken and Adams, 1996, 1997), and in Coast and Cascade populations of Douglas-fir from Washington State (Aitken et al., 1996). While an explanation for these observations is not obvious, it may be possible that the more rapidly changing spring and fall minimum daily

temperatures in the Cascades resulted in stronger stabilizing selection for bud set and budburst, and therefore, in narrower genetic variation for these traits (and the associated traits, fall and spring cold hardiness) in the Cascades. However, only in spring are coefficients of variation for AFT traits smaller in the Cascade than in the Coast population (Table 1), suggesting that only in spring are greater heritabilities for cold hardiness traits in the Cascades explained by greater selection for cold hardiness.

Strong genetic correlations for cold hardiness between fall test dates for needle and stem tissue indicate that use of a single fall test date should be adequate for ranking seedling families for fall cold hardiness. Mid-fall (October) may be best because heritabilities are largest at this time, and the peak fall frost injury risk period in the Pacific Northwest includes October (Timmis et al., 1994).

Negative genetic correlations observed between AFT injury scores in fall and spring indicate that both traits must be considered in an improvement program, in order to ensure that selection for one trait does not unfavorably impact the other. Coast populations may be more prone to such antagonism than Cascade populations, due to the more negative genetic correlation between fall and spring cold hardiness in the Coast population. These results are corroborated with similar fall–spring genetic correlations for the same families at the sapling stage ($\bar{r}_A = -0.53$ — Coast, and $-0.17 \leq \bar{r}_A \leq 0.27$ — Cascade) (Aitken and Adams, 1995a).

Moderately strong genetic correlations between tissues in AFT injury scores in both spring and fall indicate that selecting for cold hardiness using a single tissue will increase cold hardiness in the other tissues, allowing for cheaper and easier screening. Choice of the best tissue to score should consider (a) ease of measurement, (b) heritability, (c) correlations with other tissues, and (d) correlations with the economic impact of frost injury in the field. While AFT injury may be easiest to evaluate for needles, occasional needle injury is not expected to significantly impact survival, growth rate or stem form; estimated heritabilities are lower for needles than for stems and buds; and needle injury is least related to injury in other tissues.

Scoring stem and bud injury is of comparable ease, and the heritabilities of stem and bud injury are

similar. However, AFT injury to stems is more highly correlated to damage to the other two tissues than is injury to buds, and stem injury may have more serious consequences in terms of survival, although reports of mortality from cold injury are relatively infrequent. On the other hand, cold hardiness of buds may be more closely related to the impact of more frequent, non-lethal cold injury on plantation value: bud injury can cause substantial stem deformation and growth retardation (as observed in this experiment), which has cumulative negative impacts on plantation value (van der Kamp and Worrall, 1990; Reich and van der Kamp, 1993); cold injury to buds is commonly reported (van der Kamp and Worrall, 1990; Hänninen, 1991; Aitken and Adams, 1997); and buds are considerably more susceptible to cold injury in late fall than are stems or needles in seedlings (Burr et al., 1990; Guak et al., 1998; and the present study — Tables 1 and 5) and in saplings (Aitken and Adams, 1996).

4.3. Influence of soil moisture regime on selection for cold hardiness

Family rankings were fairly similar in the two moisture treatments for both fall and spring cold hardiness (Table 2). However, heritabilities were greater in the wet than the dry environment for both traits (Table 1). Strong genetic correlations between the same AFT traits in wet and dry regimes, and greater heritabilities for phenology traits in the wet regime, were also found in four Douglas-fir populations from southwestern Oregon (Kaya, 1992). Furthermore, it is easier to maintain a moist than a dry nursery soil environment over the course of a growing season. Spatial uniformity of moisture conditions is also easier to achieve in a moist than a dry soil environment. These observations indicate that selection for cold hardiness would be easier and more effective with the use of a moist soil environment.

As indicated in Section 3, drought increased mean fall cold hardiness, but had little influence on family variation for fall cold hardiness. Therefore, smaller heritabilities for fall cold injury in the dry environment may be due mainly to greater environmental variation (non-uniform drying of soil moisture) during the summer. An explanation of the persistence of

smaller heritabilities for cold hardiness in the dry regime in spring, however, remains unclear. If bud set timing influences budburst timing, then the effects of uneven drying on environmental variation in fall cold hardiness may have carried over into spring if timing of fall hardening influences timing of spring dehardening. This appears unlikely however, as chilling requirements are generally satisfied long before heat sums begin to accumulate, and environmental variation in budburst timing is therefore unlikely to be influenced by bud set timing in these populations.

4.4. Ability to predict susceptibility to natural frost injury

Genetic correlations between natural frost injury scores (FI and ABM) and fall AFT injury scores (Table 5) were moderate to strong, despite differences in year of assessment (NF in 1995, vs. AFTs in 1996), mean minimum daily fall temperatures (2.8°C warmer in September 1995 than in September 1996), age of plants (1-year-old seedlings (NF) vs. 2-year-old seedlings (AFT)), tissues scored (apical bud of leader (NF) vs. terminal bud of second-order branch (AFT)), scoring technique (percent foliage injured and proportion of apical buds killed (NF) vs. percent needle and bud injury (AFT)), and use of different personnel for scoring damage. The strength of these correlations attests to not only the consistency of family rankings for cold hardiness in natural and artificial environments, but also to the effectiveness of the AFT evaluation technique as a prediction tool for relative fall cold hardiness of families. Strong genetic correlations were also observed between Douglas-fir sapling injury due to a natural frost in early May 1992 and sapling AFT injury the following spring (Aitken and Adams, 1997). Additional evidence of the ability of the AFT technique to reliably predict cold hardiness ranking in NF events was provided by Rehfeldt (1986), who used scores from natural fall frost injury in interior Douglas-fir to validate a cold injury model developed mainly from artificial freeze tests. Cold hardiness of seedlings in AFTs was also closely related to long-term survival of Scots pine (*Pinus sylvestris*) in a harsh climate in Sweden, where survival was determined mainly by differences in fall frost hardiness (Nilsson and Walfridsson, 1995).

Regardless of the strength of the correlation between natural and artificial freeze test injury, and the similarity of heritabilities for ABM and AFT injury to buds, relying upon natural frost events in common garden tests to assess family differences in cold hardiness remains problematic, as frosts which inflict levels of injury adequate for detecting family differences are infrequent and non-uniform across sites (Campbell and Sorensen, 1973; Wheeler et al., 1990). If a NF event did provide the opportunity to evaluate cold hardiness, greater heritability for ABM than for FI (Table 5) indicates that gain in cold hardiness due to artificial selection would be greater using ABM. Furthermore, apical bud mortality is likely more important than foliage injury, because long-term plantation value is probably more strongly associated with bud survival than with needle integrity following an injurious frost.

While the levels of injury were moderate in both the NF and the AFT in November, the temperatures were considerably colder in the AFT (-22°C) than in the NF (-5°C). This points to the greater susceptibility of seedlings in their first than in their second year, and to the difficulty of predicting the absolute level of cold injury from natural frost events in the field or nursery from AFT injury scores. Nonetheless, AFT injury scores can reliably predict relative cold hardiness of different families, as indicated by the moderate to strong genetic correlations between the NF and AFT scores.

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