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Genetic Variation of Subalpine fir (Abies lasiocarpa (HOOK.) NUTT.) in the Olympic Mountains, WA, USA

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Summary

This study examines the genetic structure of subalpine fir along an elevation gradient in the Olympic Mountains, Washington. Starch gel electrophoresis was used to resolve 15 isozyme loci from 9 populations on 3 mountains. First, we describe the genetic variation of these 9 populations in the Olympic Mountains, and compare these results to those of other tree species. Then we compare genetic differentiation (G_{ST}) among populations to test if selective pressures alter genotypic frequencies in response to steep environmental gradients over the elevation range of this species. Genetic variation is relatively uniform throughout most of the sites sampled (H_o range = 0.103-0.139 in the eastern Olympic Mountains), with notably lower diversity observed at the western extent of subalpine fir's range (H_o=0.70 in the central Olympic Mountains). Lower diversity in the western Olympics may result from either founder effects as the species expanded its range from a glacial refugium, or from selection pressures on the loci considered. Genetic differentiation is greater among high elevation sites on different mountains, than among different elevation sites on the same mountain, or lower elevation sites on different mountains. Estimates of gene flow (N_m=7.8) are high among all sites.

Key words: Abies lasiocarpa, genetic diversity, gene flow, isozyme, Olympic Mountains.

Introduction

Genetic variation is high in most conifer species (HAMRICK et al., 1979; HAMRICK and GODT, 1990). While most of this variation occurs within populations, resulting in relatively uniform genetic structure among populations (TIGERSTEDT, 1973; GURIES and LEDIG, 1982), variation does occur among populations in response to environmental gradients (reviewed by LINHART and GRANT, 1996). Genetic variation in conifers has been found among stands on sites with varying water availability (STUTZ and MITTON, 1988; SHEA, 1990), elevation (GRANT and MITTON, 1977; MITTON et al., 1980; HIEBERT and HAMRICK, 1983), and slope aspect (MITTON et al., 1977), and in some cases natural selection for specific genotypes has been suggested in response to site differences (e.g., FRYER and LEDIG, 1972; MITTON et al., 1977; GRANT and MITTON, 1977; LINHART and GRANT, 1996). Differentiation of genetic structure in species occurs even when migration rates are high, suggesting that selection forces where environmental gradients are steep may be sufficient to cause genetic differentiation in spite of adequate gene flow (JAIN and BRADSHAW, 1966; GRANT and MITTON, 1977; MITTON et al., 1977, 1980; LINHART et al., 1981; WOLF and SOLTIS, 1992). In a review of the evolutionary significance of genetic differentiation in plants, LINHART and GRANT (1996) conclude that environmental heterogeneity generates genetic heterogeneity by causing differing selection pressures and creating significant barriers to gene flow through differences in phenology.

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Subalpine fir (*Abies lasiocarpa* (HOOK.) NUTT.) is a common subalpine tree species across much of North America (*Figure 1* – FOWELLS, 1965). Subalpine fir in the Olympic Mountains (*Figure 1*) have lived in relative isolation from other subalpine fir populations to the north on Vancouver Island, Canada, and to the east in the Cascade Mountains since 11,500 to 9,000 years BP when a low elevation subalpine parkland most likely connected these populations (BARNOSKY et al., 1981; WHITLOCK, 1992). Subalpine fir existed in glacial refugia in the eastern Olympic Mountains at low elevations and probably also as small isolated populations at high elevations (i.e. on nunatuks) throughout the eastern Olympic range (e.g. Moose lake pollen and macrofossil data shows subalpine fir at 1500 m from 13,000 years BP – BRUBAKER and MCLACHLAN, 1996).

In the Olympic Mountains, subalpine fir forests currently dominate most drier slopes in the north-eastern portion of the range, from higher elevations near treeline (~ 1800 m) to mixed-montane forests at lower elevations (~ 1300 m – ETTL and PETERSON, 1995). Subalpine fir is the dominant tree species at high elevations where it typically grows in scattered clumps. Subalpine fir is also dominant in the middle of its elevation range, where it grows in association with lodgepole pine (*Pinus contorta* DOUGL.), Douglas-fir (*Pseudotsuga menziesii* (MIRBEL) FRANCO), Alaska yellow-cedar (*Chamaecyparis nootkatensis* D. DON), and western white pine (*Pinus monticola* DOUGL.) on drier sites; Alaska yellow-cedar, mountain hemlock (*Tsuga mertensiana* (BONG.) CARR.), and Pacific silver fir (*Abies amabilis* DOUGL.) are common associates on wetter sites. Subalpine fir shares canopy dominance with these respective tree species at subalpine fir's lower elevation ecotone. Subalpine fir is less common below 1300 m, although it is found in cold air drainages down to 300 m.

In a previous study, we quantified the effects of climate on radial growth across the elevation range of subalpine fir including both the upper- and lower-elevation ecotones in the Olympic Mountains, Washington (ETTL and PETERSON, 1995). This study examines the genetic structure of subalpine fir populations along this environmental gradient, as well as among mountains. Although subalpine fir is currently widespread throughout the drier subalpine zone within the Olympic Mountains, we suspect that subalpine fir may have relatively limited genetic diversity compared to other conifers due to isolation from other populations of subalpine fir at the beginning of the Holocene. We also explore the possibility of genetic dif-

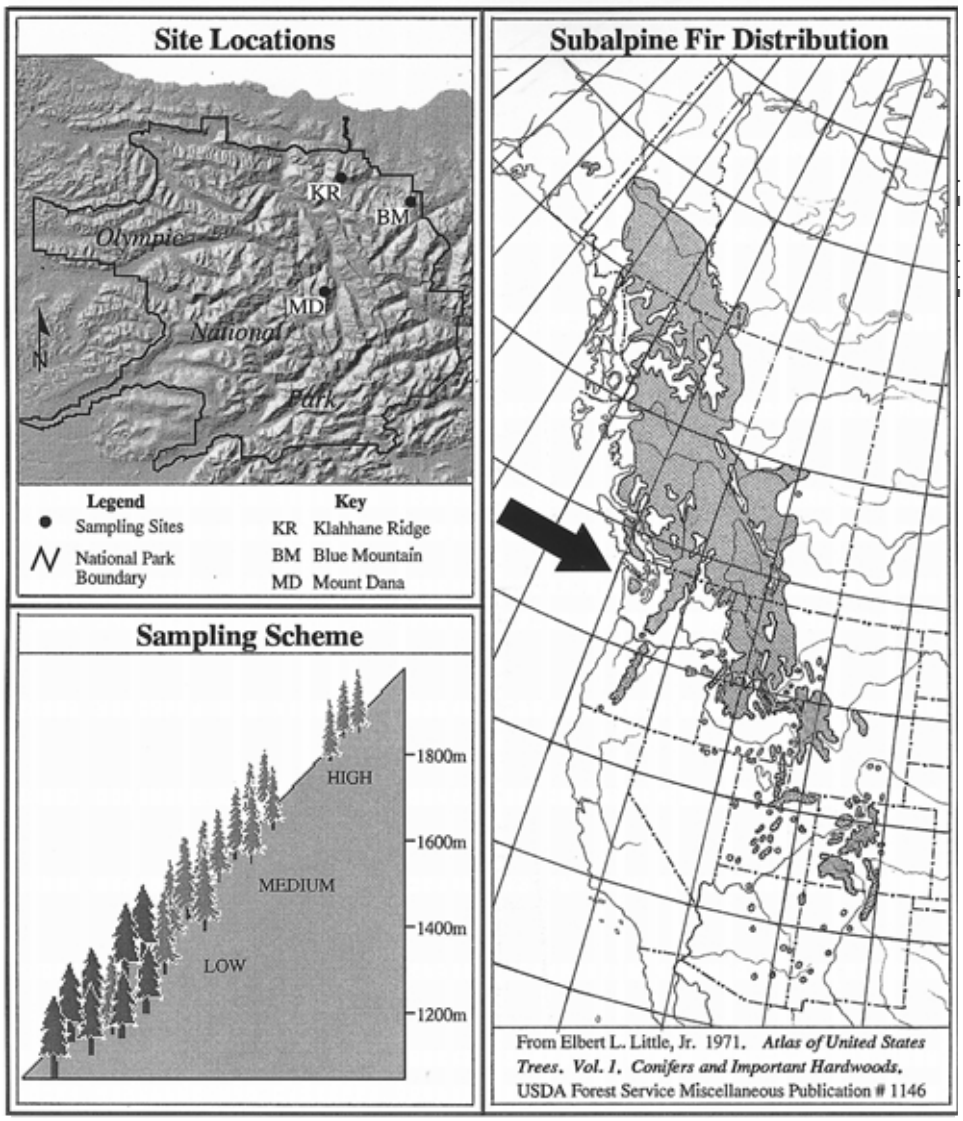

Figure 1. – Geographic distribution of *Abies lasiocarpa* in North America.

ferentiation along an elevation gradient. Little is known about the genetic variation or structure for subalpine fir, however GRANT and MITTON (1977) found dramatic differentiation in peroxidase among 3 different morphologies (spire, flag, and krummholz) at Niwot Ridge in the Rocky Mountains, USA. SHEA (1990) also working at Niwot Ridge found genetic differentiation between wet and dry sites and among different age cohorts of subalpine fir. This study is the first to describe genetic variation of subalpine fir in the Pacific Northwest, USA.

Methods

Data Collection and Processing

Subalpine fir populations were sampled at 3 elevations on southwest-facing slopes on Klahhane Ridge and Blue Mountain: the upper (treeline 1800 m), middle (1575 m), and lower (1350 m) extent of its elevation range (*Figure 2*, ETTL and PETERSON, 1995). These two peaks, separated by 14 km, are at the relatively dry end of the local geographic range of subalpine fir in the Olympic Mountains (*Figure 3*, ~125 cm/yr mean annual precipitation, PHILLIPS and DONALDSON, 1972). At least 50 trees were sampled from 20 m-wide band transects at each site. In addition, 30 trees were sampled from open-grown or dominant trees at a relatively wet treeline site at Mount Dana (~350 cm/yr mean annual precipitation, PHILLIPS and DONALDSON, 1972), the westernmost extent of subalpine fir in the Olympic Mountains; approximately 22 km to the southwest of the two drier sites (ETTL and PETERSON, 1995). Trees at middle (1600 m) and low elevations (1350 m) on Blue Mountain were sampled from 2 sites (separated by approximately 200 m at each elevation) in order to minimize differences in slope and aspect (hereafter Blue Mountain low-1 and low-2, middle-1 and middle-2). This study was conducted along with a dendroecological study of subalpine fir (ETTL and PETERSON, 1995) and a comparison of growth rate to multi-locus heterozygosity (ETTL, 1995), and therefore sites were selected to maintain constant slope and aspect; only mature dominant and codominant trees (after SPURR and BARNES, 1980) without major stem or crown deformities were selected. The selection of dominant and codominant trees minimizes radial growth variation due to competition from neighboring trees and this was absolutely critical for growth rate with multi-locus heterozygosity (ETTL, 1995).

A 15 cm to 20 cm section of shade foliage was clipped from the lower branches on the north-facing side of each sample tree. The cuttings were placed on ice and kept cool to suspend biological activity until processing. Needles were initially ground with a mortar and pestle in liquid nitrogen and 0.1 ml of buffer (0.1 M Tris-HCl pH= 8.0, 12% PVP-40 (w/v), 2% sucrose (w/v), 10 mM DL-dithiothreitol, and < 0.01% bovine serum albumin [fraction V]). The buffered leaf extracts were stored in microtiter trays in an ultralow temperature freezer (–70°C). Samples were subsequently stored on dry ice and flown to the USDA Forest Service Institute of Forest Genetics lab in Albany, California, where isozyme analysis was conducted.

Isozyme and Statistical Analysis

Leaf tissue solutions were subjected to electrophoretic separation on starch gels using techniques of CONKLE et al. (1982) and MILLAR (1985), and gel systems of STRAUSS and CONKLE (1986) and WENDEL and WEEDEN (1989). An initial screening was performed to determine enzyme activity for 33 different enzymes on 6 different gel systems (ETTL, 1995). Fifteen loci from 12 enzyme systems showed strong and repeatable resolution: aspartate aminotransferase (*Aat-1* and *Aat-2*), fructose-biphosphate aldolase (*Ald*), fructose-1,6-diphosphate (*Fdp*), ᴅ-D-galactoside galactohydrolase (*Gal*), isocitrate dehydrogenase (*Idh*), malate dehydrogenase (*Mdh-1* and *Mdh-2*), peptidase (*Pep*), peroxidase (*Per*), phosphoglucoisomerase (*Pgi-1* and *Pgi-2*) phosphoglucomutase (*Pgm*), 6-phosphogluconate dehydrogenase (*Six*), and UDP-glucose pyrophosphotase (*Ugp*). These loci were scored on 5 different gel systems. Diploid genotypes were interpreted from segregation patterns and comparison of isozyme phenotypes of the same enzymes for related species (DIANE DELANY, USDA Forest Service, Albany, CA, personal communication).

Allele frequencies, mean number of alleles per locus, percentage of polymorphic loci (alleles were classified as polymorphic if more than 1 allele was observed), and mean heterozygosity levels (computed as both observed [H_o] and HARDY-WEINBERG expectations [H_e]) were calculated for each site as measures of genetic diversity (NEI, 1978). Deviations from HARDY-WEINBERG equilibrium were assessed with Chi-square analysis (WORKMAN and NISWANDER, 1970); allele frequencies were pooled when frequencies of some classes were low, and YATE's correction for continuity was applied. Subdivision of genetic structure among sites was assessed with Chi-square tests for differences of allele frequencies, *F*-statistics (WRIGHT, 1965; NEI, 1973, 1977, 1986), and NEI's unbiased genetic distance (calculated with exclusion of 6 monomorphic loci, NEI, 1978). Chi-square analyses of allele frequencies and G_{ST} values (the proportion of interpopulation differentiation) were compared both by elevation for mountains (G_{KR}, G_{BM}) and between different mountains for all elevations (G_{HIGH}, G_{MID}, G_{LOW}). Estimates of gene flow were calculated from F_{ST} values (equivalent to G_{ST}), N_m=1/4(1/F_{ST}-1), where N_m represents the number of migrants exchanged among populations per generation (WRIGHT, 1951; SLATKIN and BARTON, 1989). Unweighted pair-group method (UPGMA) cluster analysis (SNEATH and SOKAL, 1973) was used to visualize differences in genetic distance among groups. All analyses were performed with the programs BIOSYS-1 (SWOFFORD and SELANDER, 1989) and GeneStat-PC (LEWIS and WHITKUS, 1993). HAMRICK and GODT's (1990) H_{es} (the average genetic heterozygosity over all loci) was used to compare heterozygosity levels in subalpine fir with those of other conifers.

Results

Genetic Variation within Populations

Six of the fifteen loci resolved were monomorphic (*Ald, Fdp, Gal, Mdh-2, Pep,* and *Pgi-1*), five had relatively low variability (*Aat-1, Aat-2, Idh, Mdh-1*, and *Six*), and four had relatively high variability (*Per, Pgi-2, Pgm,* and *Ugp – Table 1*). Genetic variability is similar among all sites on Blue Mountain and Klahhane Ridge, but notably lower at the Mount Dana site (*Table 2*). Observed mean observed heterozygosity ranges from H_o=0.103 to 0.139 on Blue Mountain and Klahhane Ridge, but is only 0.070 at Mount Dana. The percentage of polymorphic loci was also substantially lower at Mount Dana (26.7%) than at the other sites (46.7% to 60%). This lower percentage of polymorphic loci is explained by the fact that Mount Dana loci are fixed for *Aat-2, Per, Mdh-1, Six* and *Idh.* In addition, Mount Dana has substantially lower frequency of the *Pgm-1* allele (*Table 1*) than sites on Klahhane Ridge or Blue Mountain. For all sites the most variable loci also show relatively high levels of heterozygosity (*Pgi-2,* H_s=0.7397; *Pgm,* H_s=0.3533; *Ugp,* H_s=0.2836; and *Per,* H_s=0.2151), with 5 alleles observed for both *Pgi-2* and *Ugp,* and relatively equal distribution among the 5 alleles for *Pgi-2* at most sites.

Inbreeding coefficients (F_{IS}) estimate the deviations from HARDY-WEINBERG equilibrium of individuals in each sample subpopulation (i.e. at each of the 9 sites sampled in this study),

Table 1. – Estimated allele frequencies of allozymes for 9 populations of subalpine fir. Populations are abreviated by mountain and elevation, Mount Dana (MD), Klahhane Ridge (KR), and Blue Mountain (BM) and elevation high (h), middle (m), low (l). Note 2 sites were sampled at middle (BMm1 and BMm2) and low elevations (BMl1 and BMl2) on Blue Mountain.

	MDh	KRh	KRm	KRlw	BMh	BMm1	BMm2	BMl1	BMl2
AAT1									
(N)	31	47	51	45	57	19	33	35	21
1	.032	.032	.020	.000	.009	.026	.015	.014	.048
2	.935	.883	.961	.978	.982	.947	.924	.971	.905
3	.032	.085	.020	.022	.009	.026	.061	.014	.048
AAT2									
(N)	31	47	51	45	57	19	33	35	21
1	.000	.000	.010	.011	.009	.000	.000	.057	.024
2	1.000	1.000	.990	.989	.991	.974	1.000	.943	.976
3	.000	.000	.000	.000	.000	.026	.000	.000	.000
ALD									
(N)	19	34	57	21	33	46	45	51	31
1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
FDP									
(N)	19	31	55	21	33	28	45	51	27
1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
GAL									
(N)	2	35	57	21	33	47	45	51	31
1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
IDH									
(N)	31	47	50	45	57	19	33	35	21
1	.000	.106	.040	.033	.009	.026	.061	.100	.024
2	1.000	.894	.960	.944	.991	.974	.939	.900	.976
3	.000	.000	.000	.022	.000	.000	.000	.000	.000
MDH1									
(N)	31	47	51	45	57	19	33	35	21
1	.000	.000	.020	.067	.000	.000	.015	.043	.024
2	1.000	1.000	.980	.933	1.000	1.000	.985	.957	.976
MDH-2									
(N)	19	35	57	21	33	47	45	51	31
1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
PEP									
(N)	18	35	57	21	33	47	45	51	31
1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
PER									
(N)	31	47	49	45	57	18	33	34	21
1	1.000	.915	.827	.822	.851	.889	.864	.882	.810
2	.000	.085	.173	.178	.149	.111	.136	.118	.190
PGI1									
(N)	30	45	51	45	57	18	33	35	21
1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
PGI2									
(N)	30	45	51	45	57	18	33	35	21
1	.417	.256	.196	.200	.193	.278	.136	.143	.262
2	.133	.122	.294	.233	.202	.167	.303	.143	.143
3	.333	.422	.235	.256	.395	.278	.212	.386	.190
4	.100	.200	.225	.178	.175	.222	.333	.300	.333
5	.017	.000	.049	.133	.035	.056	.015	.029	.071
PGM									
(N)	31	46	44	42	57	19	33	35	21
1	.081	.391	.330	.226	.158	.158	.227	.300	.357
2	.919	.609	.670	.774	.842	.842	.773	.700	.643
SIX									
(N)	31	47	49	45	57	19	33	35	21
1	1.000	.979	.969	.978	.982	1.000	1.000	.986	1.000
2	.000	.021	.031	.022	.018	.000	.000	.014	.000
UGP									
(N)	31	47	49	45	57	19	33	35	21
1	.000	.000	.020	.044	.026	.026	.015	.043	.048
2	.000	.053	.010	.044	.018	.026	.015	.043	.000
3	.016	.064	.133	.067	.123	.105	.076	.057	.095
4	.887	.862	.765	.800	.798	.842	.879	.857	.857
5	.097	.021	.071	.044	.035	.000	.015	.000	.000

and F_{IS} values are small for most polymorphic loci (*Table 3*). Chi-square tests indicate that *Aat-1* has a significant deficiency of heterozygotes at all high-elevation sites (Blue Mountain F_{IS} =0.493, c^2=14.36, p<0.001; Klahhane Ridge F_{IS} =0.197, c^2=7.28, p=0.007; Mount Dana F_{IS} =0.475, c^2=17.45, p<0.001). Blue Mountain low-1 elevation and middle-2 elevation sites

Table 2. – Genetic variability statistics for 9 subalpine fir sites.

Population	Mean sample size/locus	Mean # of alleles/locus	Percentage of loci polymorphic*	Mean heterozygosity	
				observed	expected
Mount Dana high	30.7 (.3)	1.6 (.3)	26.7	.070 (.042)	.079 (.048)
Klahhane Ridge high	45.5 (1.3)	1.8 (.3)	46.7	.139 (.057)	.137 (.055)
Klahhane Ridge middle	50.1 (.5)	2.1 (.3)	60.0	.138 (.056)	.145 (.059)
Klahhane Ridge low	44.8 (.2)	2.1 (.3)	60.0	.139 (.057)	.143 (.058)
Blue Mountain high	56.9 (.1)	2.0 (.4)	53.3	.108 (.054)	.115 (.054)
Blue Mountain middle-1	17.7 (1.1)	1.9 (.3)	46.7	.116 (.054)	.117 (.055)
Blue Mountain middle-2	33.0 (.0)	1.9 (.4)	46.7	.103 (.043)	.124 (.053)
Blue Mountain low-1	34.6 (.3)	2.0 (.3)	60.0	.120 (.046)	.139 (.053)
Blue Mountain low-2	21.0 (.0)	1.9 (.3)	53.3	.130 (.059)	.143 (.059)

*) Loci were classified as polymorphic if more than one allele was observed.

had deficiencies in the number of heterozygous individuals (approaching statistical significance, p=0.10), and excesses in rare homozygotes for *Pgm* (F_{IS} =0.388, c^2=3.92, p=0.048; F_{IS} =0.396, c^2=3.50, p=0.061 respectively). Differences from HARDY-WEINBERG expectations were also observed for *Pgi-2* at the Blue Mountain low-1 elevation site (F_{IS} =0.087, c^2=5.93, p=0.015). However, there is an excess in both common and rare homozygotes for this locus. The 6 significant differences from HARDY-WEINBERG expectations (out of 81 tests – 9 polymorphic loci, 9 sites) is less than the number expected due to type-1 statistical error (with p =0.10, ~8 significant differences are expected). However, the consistent excesses and deficiencies in heterozygotes among sites (particularly for *Aat-1* at all high-elevation sites) suggest that these differences are probably real.

Genetic Variation among Populations

Genetic distance estimates indicate little differentiation of genetic variation among the sites on Blue Mountain and Klahhane Ridge (*Figure 2*), and genetic distances among these 8 sites are low (0.000 to 0.009). Furthermore, only somewhat larger differences exist between Mount Dana and all other sites (0.001 to 0.017), however the genetic structure of subalpine fir on Mount Dana is clearly different than all the sites on Blue Mountain and Klahhane Ridge. Estimation of *F*-statistics for all of the sites as individual populations (F_{ST} values *Table 3*) indicate that 97% of the genetic variation is within sites, while

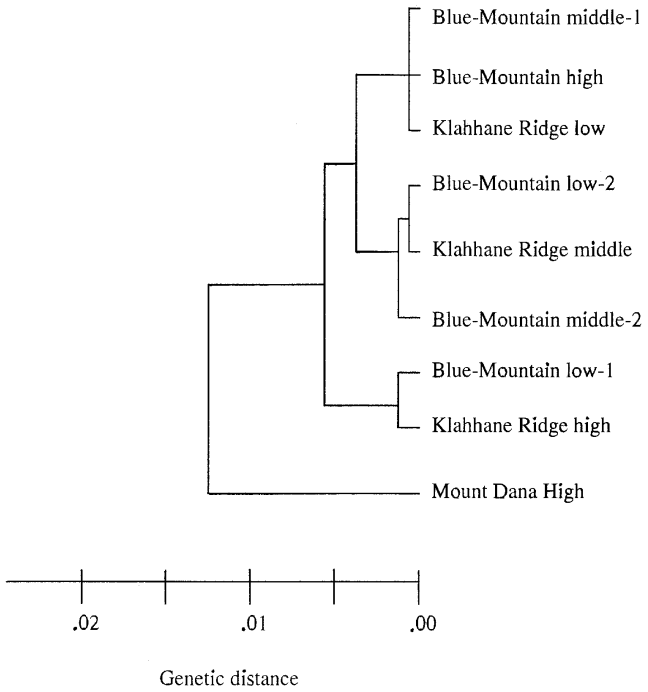

Figure 2. – UPGMA cluster analysis of 9 subalpine fir sites in the Olympic Mountains using NEI's (1978) unbiased genetic distance estimates from 9 polymorphic loci.

Table 3. – Measures of genetic substructure for subalpine fir among elevations and mountains. F_{ST} (or G_{ST}) is the proportion of genetic variation among all 9 populationgs (NEI, 1973), F_{IS} is the fixation index measuring deviations from random mating, G_{high} is the proportion of genetic variation among the 3 high elevation sites, $G_{BMKRHIGH}$ is proportion of genetic variation among the high elevation sites on Blue Mountain and Klahhane Ridge, G_{MID} and G_{LOW} are the proportion of genetic variation among middle and low elevation sites on Blue Mountain and Klahhange Ridge together, G_{BM} and G_{KR} are the proportion of genetic variation among sites on Blue Mountain and Klahhane Ridge. All G_{ST} values are unbiased for sample size and population number (NEI's, 1986). N_m is an estimate of the number of migrants exchanged among sites per generation (SLATKIN and BARTON, 1989).

Locus	F_{ST}	F_{IS}	G_{HIGH}	$G_{BMKRHIGH}$	G_{MID}	G_{LOW}	G_{BM}	G_{KR}	N_m
AAT1	0.016	0.158	0.0208	0.0519	0.0000	0.0107	0.0000	0.0264	15.4
AAT2	0.023	-0.036	0.0000	0.0000	0.0009	0.0024	0.0083	0.0000	10.6
PGI2	0.032	0.018	0.0159	0.0000	0.0038	0.0166	0.0129	0.0208	7.6
UGP	0.015	-0.030	0.0160	0.0063	0.0016	0.0000	0.0000	0.0060	16.4
PER	0.028	0.124	0.0646	0.0100	0.0000	0.0000	0.0000	0.0113	8.7
PGM	0.052	0.161	0.1390	0.1190	0.0235	0.0040	0.0248	0.0207	4.6
MDH1	0.026	-0.046	*****	*****	0.0000	0.0000	0.0025	0.0312	9.4
SIX	0.011	-0.023	0.0000	0.0000	0.0140	0.0000	0.0000	0.0000	22.5
IDH	0.029	-0.004	0.0800	0.0742	0.0000	0.0129	0.0143	0.0128	8.4
mean	0.031	0.054	0.0477	0.0352	0.0042	0.0061	0.0080	0.0166	7.8

only 3% is among sites. A comparison of *F*-statistics for subdivision of genetic variation among elevations and mountains (F_{ST} or G_{ST}) indicates that the majority of genetic variation among sites is the result of differences among high elevation sites (*Table 3*). There is almost no genetic variation among all of the low elevation sites (G_{LOW}=0.0061) and all of the middle elevation sites (G_{MID}=0.0042). The largest differences occur among all high-elevation sites (G_{HIGH}=0.0477). This is not surprising considering the lower overall diversity of the Mount Dana site when compared to Blue Mountain and Klahhane Ridge high-elevation sites (*Table 2*), however differences among Blue Mountain and Klahhane Ridge high-elevation sites are also relatively large ($G_{BMKRHIGH}$=0.0352), suggesting that greater differentiation in genetic structure has occurred among high elevation sites. Variation among different elevations on the same mountain are negligible at Blue Mountain (G_{BM}=0.0080), however are larger at Klahhane Ridge (G_{KR}=0.0166), indicating greater differentiation in genetic structure with elevation on Klahhane Ridge.

Chi-square analysis of allele frequencies shows significant differences in allele frequencies among all sites, and groups of sites (both by mountain and elevation, *Table 4*). Significant differences were found for comparisons of allele frequencies among all sites for most loci (note exceptions, *Aat-1* and *Six-1*). The largest differences in allele frequencies were observed between high elevation sites on different mountains. This is true even when the less diverse Mount Dana site is excluded from calculations (i.e., comparison of Blue Mountain and Klahhane Ridge high elevation sites).

Differences in allele frequencies among elevations were also observed. There are significant differences in allele frequencies among all elevations on each mountain (Blue Mountain and Klahhane Ridge). However, allele frequencies among middle- and low-elevation sites from the same elevation on different mountains, and among different elevations (excluding high-elevation sites) on the same mountains, show almost no significant differences in allele frequencies (except *Pgi-2* among all low elevation sites, c^2=17.19, p=0.028). That is, the differences in allele frequencies among elevations is primarily due to differences between high elevation sites and all lower sites.

Discussion

Comparing Genetic Diversity of Related Taxa

The levels of genetic diversity estimated for subalpine fir in this study (H_{es} mean=0.129 [std.=0.055]), are generally lower than those observed for other conifers (H_{es} mean=0.173 [std.=0.011], HAMRICK and GODT, 1990). There is also considerably less variation among the sites sampled in this study (G_{ST}=0.031) than levels reported for other conifers (G_{ST}=0.068 [0.013], HAMRICK and GODT, 1990). Levels of heterozygosity for subalpine fir in the Olympic Mountains are also lower than those reported for other *Abies* species: *A. alba* MILL. (mean H_e=0.149, 17 loci), *A. bornmuelleriana* MATTF. (mean H_e=0.161, 17 loci), *A. cephalonica* LOUD. (mean H_e =0.161, 17 loci) (FADY and CONKLE, 1993). Other estimates of heterozygosity in *Abies* species using only polymorphic loci have found (not surprisingly) even higher levels of heterozygosity. DIEBEL and FERET (1991) found high levels of heterozygosity in *A. fraseri* (PURSH) POIR (mean H_e =0.258 for 4 loci), as did NEALE and ADAMS (1985) for *A. balsamea* (L.) MILL. (range: 0.261 to 0.292, 8 loci). In comparison, heterozygosity for only polymorphic loci of subalpine fir in the Olympic Mountains (range =0.117 to 0.232) are also lower than either *A. fraseri* or *A. balsamea*. The genetic diversity observed in this study is probably biased by our inclusion of only dominant and codominant trees, most likely leading to overall higher estimates of genetic diversity because tree populations often show increasing genetic diversity through time due to selection for outcrossed offspring (LEDIG, 1986).

At least one other isozyme study has been used to describe the genetic diversity of subalpine fir (SHEA, 1990; although see GRANT and MITTON, 1977). SHEA (1990) described the genetic structure of subalpine fir in two adjacent sites (one wet, one dry) in the Front Range of the Colorado Rocky Mountains. Mean observed heterozygosity is 0.081 for these sites and 44.4% of the loci sampled were polymorphic. These levels of genetic variability are considerably less than those observed for subalpine fir at the Blue Mountain and Klahhane Ridge sites, but slightly higher than those observed at the Mount Dana site. The low levels of genetic variability reported by SHEA (1990) may result from relatively large amounts of inbreeding among the sampled trees; the observed inbreeding coefficients (mean F_{IS}=0.341) support this claim. Inbreeding coefficients for subalpine fir in the Olympic Mountains are much closer to zero (mean F_{IS}=0.054), suggesting that inbreeding is not as important a determinant of the genetic structure of subalpine fir as it is in the Rocky Mountains.

It is difficult to compare the genetic variation observed in the Olympic Mountains to that described by SHEA (1990), because sample sites in the Rocky Mountains were separated by only 100 meters, and this sample may not be comparable to samples of subalpine fir across a broader geographic range. However, HAMRICK and GODT (1990) have suggested that samples of genetic variability from populations of a species are often

Table 4. – Comparison of allele frequencies among all sites, sites on Klahhane Ridge, sites on Blue Mountain, and high elevation sites on Klahhane Ridge and Blue Mountain.

	All			Klahhane Ridge			Blue Mountain			Klahhane Ridge and Blue Mountain High		
Locus	χ^2	df	prob	χ^2	df	prob	χ^2	df	prob	χ^2	df	prob
AAT1	19.42	16	.247	9.45	4	.051	8.27	8	.408	8.92	2	.012
AAT2	32.94	16	.008	.998	2	.607	16.19	8	.040	.83	21	.362
PGI2	83.30	32	.000	28.09	8	.000	26.55	16	.047	6.22	14	.185
UGP	44.96	32	.064	13.55	8	.094	11.99	16	.745	6.87	4	.143
PER	16.37	8	.037	4.13	2	.127	1.49	4	.828	2.00	1	.158
PGM	33.07	8	.000	5.60	2	.061	10.31	4	.036	14.34	1	.000
MDH1	19.01	8	.015	7.92	2	.019	6.15	4	.188	–	–	–
SIX	5.46	8	.707	.210	2	.900	2.45	4	.653	.04	1	.845
IDH	33.66	16	.006	9.67	4	.046	10.03	4	.040	9.80	1	.002
Total	288.18	144	.000	79.62	34	.000	93.43	68	.022	49.01	15	.000

representative of the diversity over a much larger area. If estimates of genetic diversity for subalpine fir in Colorado are representative of the diversity of subalpine fir in the Rocky Mountains on the whole, then differences between diversity estimates in the Rockies and the Olympics are intriguing but difficult to explain. Subalpine fir occupies a broad range across western North America (*Figure 1*), and only a comprehensive study of subalpine fir across its entire range would provide answers to differences observed between the Rockies and the Olympics.

Subalpine Fir Expansion in the Olympic Mountains

Paleoecological studies suggest that subalpine fir populations in the Cascades Mountains, Fraser River Valley, B.C., and Olympic Mountains may have originated from a relatively continuous population following deglaciation (CRITCHFIELD, 1984). Recent pollen data from a high elevation site in the north-eastern Olympics (Moose Lake, 1500 m) provides evidence that subalpine fir also existed in nunatuks at high elevations (BRUBAKER and MCLACHLAN, 1996). BUCKINGHAM et al. (1995) and PETERSON et al. (1997) interpret these pollen records as indicating that most of the current high-elevation tree species have occupied these sites since 10,000 BP, suggesting that subalpine fir populations may have expanded across their current geographic range from high and/or low elevation refugia.

The relatively low levels of diversity observed for subalpine fir in this study may be the result of expansion of subalpine fir populations after isolation. The relatively lower diversity at Mount Dana (the westernmost extent of subalpine fir within the Olympic Mountains) supports the idea that subalpine fir has expanded its range from the northeast toward the west since the last glacial maximum, although the possibility that the Mt. Dana population of subalpine fir originated from a more westerly refugia near or on Mt. Dana can not be discounted. One interpretation of the low genetic diversity observed at Mount Dana is that this population originated from a limited number of individuals of subalpine fir as populations expanded 13,000 to 10,000 years BP. Pollen records indicate that subalpine fir was already present at high elevations (at Moose Lake, 1500 m) in the northeastern Olympics as early as 13,000 years BP (BRUBAKER and MCLACHLAN, 1996), and therefore subalpine fir probably has had ample opportunity to expand its range westward following deglaciation.

The Olympic Mountains create a rain shadow effect that results in considerable differences in snowpack among westerly sites (i.e. Mt. Dana, ~325 cm/year), and more easterly sites (Blue Mountain and Klahhane Ridge, ~125 cm/year). Gene flow between the Mount Dana population and more easterly populations of subalpine fir is probably limited, because heavier snowpacks at Mount Dana results in later maturation of pollen cones therefore limiting pollen transfer to female cones on drier more easterly sites. GRANT and MITTON (1977) found substantial genetic differentiation along an elevation gradient (especially at treeline) in the Rocky Mountains for both subalpine fir and ENGELMANN spruce (*Picea engelmannii* PARRY). The lower diversity at Mount Dana may also be explained as resulting from selection for individuals with traits that are adapted to heavy snowpacks and the short growing seasons at Mount Dana, and this selection could result in lower genetic diversity. Regardless of the initial cause of lower genetic diversity at Mt. Dana (either a small initial population or selection for survival in a more snow-dominated environment) lower genetic diversity could be maintained at Mt. Dana if gene flow is limited and alleles are lost through genetic drift. Interpretations of the lower genetic diversity observed at Mount Dana could be improved by describing the genetic structure of subalpine fir on additional high-elevation, high-snowfall sites in the Olympic Mountains.

Genetic Differentiation with Elevation

Differences in allele frequencies among high-elevation sites may result from founder events or differences in outcrossing rates of high elevation sites. There are significant differences in allele frequencies among high elevation sites on different mountains, and between high elevation and lower elevation sites on the same mountain; there are no significant differences among middle and low elevation sites on the same or on different mountains (*Table 4*). The high and low elevation sites are separated horizontally by about 1 km on both Klahhane Ridge and Blue Mountain, and although most pollen falls within 250 m of the source for many plants (LEVIN and KERSTER, 1974), some pollen is likely transported greater distances, particularly in wind pollinated trees (MUONA, 1990). Snow melts as much as a month later at high elevation sites than at low elevation sites (ETTL and PETERSON, 1995; WOODWARD et al., 1995) and differences in phenology among these sites may be sufficient to prevent pollen exchange among lower and high elevation populations. In contrast, low and middle elevation sites from Klahhane Ridge and Blue Mountain are separated by 14 km, however there is no genetic differentiation among these sites. Subalpine fir is found primarily at higher elevations (above 1300 m) and does not form a continuous range between Klahhane Ridge and Blue Mountain (although subalpine fir does extend down to lower elevations in cold air drainages), and synchronous dispersal of pollen among these sites may be sufficient to allow panmictic breeding. Seed dispersal in subalpine fir is poorly understood, however the majority of seed has been shown to fall within 30 m of mature trees (NOBEL and RONCO, 1978). Thermal upslope winds are important in transporting seed to higher elevations (ALEXANDER et al., 1990), and limited thermal wind events may lead to founder events near treeline – explaining some of the genetic differentiation among high elevations.

Subalpine fir often grow in discrete clumps near treeline in the Olympic Mountains, and there is physical evidence (ramets formed from layering of branches) that these clumps have expanded in size through time, suggesting smaller initial populations. In one study of subalpine fir clumps in the Olympics, 30% of the stems arose from vegetative layering (ED SCHREINER, personal communication, unpublished data, USGS Biological Resources Division, Port Angeles, WA). The sampling of subalpine fir from clumps at high elevation sites may explain differences in genetic structure between high elevation sites. If individuals growing in the same clump have originated from layering of branches of neighboring trees, then the effective sample sizes at high elevations may be smaller than indicated, because multiple sampling of clones of the same individual may have occurred (3 to 5 individuals were sampled from clumps). An examination of allele frequencies from sampled clumps at each of the high elevation sites, suggests that in at least one instance individuals sampled may in fact be clones. For example, 3 trees sampled from one tree clump at Klahhane Ridge all have the genotype $Aat\text{-}1_{13}$, $Pgi\text{-}2_{13}$, and Pgm_{12}. The $Aat\text{-}1_{13}$ genotype is expected to be very rare (frequency of alleles: 1=0.032, 3=0.085) at Klahhane Ridge (*Table 1*), so it is unlikely that the 3 random individuals would have this genotype. Similar genotypic patterns between groups of clumped trees are observed for other clumps at each high elevation site, but in most cases individuals sampled from each clump are genetically different. Clonal reproduction of subalpine fir at high elevation sites may also explain some of the genetic differentiation of these sites.

Differentiation among high elevation sites may also be due to selection. Gene flow among all sites is high (N_m=7.8) and most likely is sufficient to limit genetic differentiation through genetic drift at neutral loci (i.e. N_m >1, LEVIN and KERSTER, 1974; SLATKIN and MARUYAMA, 1975). In this study, sites were sampled in order to maximize environmental constraints at both the upper and lower elevation ecotone for subalpine fir, and therefore stronger genetic differentiation at both ecotones, than at the more mesic sites, could be expected if selection were acting on the genetic structure of populations. Previous studies of growth-climate relationships of subalpine trees, indicates that climate is most strongly limiting at treeline (FRITTS, 1976; TRANQUILLINI, 1979; VILLALBA et al., 1994; ETTL and PETERSON, 1995). Therefore our observation of genetic differentiation only at higher elevations (but not the low-elevation ecotone) is consistent with the idea that selection could result from strong environmental pressures at treeline, with genetic differentiation moderated by gene flow from lower elevations (SLATKIN, 1973; ENDLER, 1977). However, founder effects, differences in outcrossing rates among sites, and clonal reproduction near treeline are probably sufficient to explain the observed genetic differentiation.

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Genotypic and Environmental Variation of *Castanea crenata x C. sativa* and *Castanea sativa* Clones in Aptitude to Micropropagation

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Summary

Thirty five selected chestnut clones were micropropagated in six culture media. Data about the *in vitro* multiplication stage are presented: clonal variation, effect of culture media, interaction between both factors, variance components, clonal repetitivities and correlations between pairs of traits. Highly significant differences were found for all traits assessed. The genotype influence was the more important factor in all traits related with multiplication rates in clonal propagation. The high values of the clonal repetitivities indicate that these characteristics are genotype dependents and allow the prediction of the behaviour of the clones when one wants to start with *in vitro* propagation. High variability in growth rates and behaviour was found depending on the clone, so that multiplication coefficient ranged between 8.45 and 1.96 for hybrid clones. *Castanea sativa* clones have middle or low multiplication rates in comparison with hybrid clones. For shoot apex necroses results showed an important weight for the factor culture medium, the most favorable medium to avoid necrosis is GD; high levels of NH_4^+ and low levels of NO_3^-, HPO_4^{2-}, K^+, Mg^{2+} and NO_3^-/NH_4^+ ratio have a favourable influence, and there is very limited influence of the calcium concentration on the expression of shoot apex necrosis. Two types of necrosis have been observed, the first one "limited to apex" that allows to continue the growth of the explant by induction of branching and a second type "descending" for other clones that gradually extend down to the shoot and deteriorate it completely. For chlorosis results showed an important weight for the factor culture medium and also for interaction genotype-culture medium. The most favourable medium to avoid chlorosis is MS ($\frac{1}{2}NO_3$) and the worse medium is SH, low concentrations of HPO_4^{2-}, K^+, NO_3^-, and NO_3^-/NH_4^+ ratio and high levels of NH_4^+ promote absence of chlorosis. Correlations between micropropagation variables show that it is possible to select pairs of production traits and at the same time to improve against the appearance of apical necrosis.

Key words: micropropagation, *Castanea crenata x C. sativa*, *Castanea sativa, Castanea mollissima x C. sativa* clones, multiplication rates, clonal repetitivity, variance components, apical necrosis, chlorosis, nitrate:ammonium ratio.

Introduction

Chestnut is a species planted for quality wood production in acid, medium or good quality soils. Hybrid clones of *Castanea sativa* MILL. with *C. crenata* SIEB. et ZUCC. or *C. mollissima* BLUME resistant to ink disease, caused by several *Phytophthora* species, are recommended for plantations in areas affected by