USDA FOREST SERVICE
NATIONAL FOREST GENETICS LABORATORY
(NFGEL)

Annual Report 2003 – 2004
(FY04)

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INTRODUCTION

This report covers laboratory activities and accomplishments during Fiscal Year 2004.
October 1, 2003 through September 30, 2004

Background

NFGEL was established in 1988 as part of the National Forest System of the USDA-Forest Service. The focus of the lab is to address genetic conservation and management of all plant species using a variety of laboratory techniques including DNA analyses. NFGEL services are provided to managers within the Forest Service, other government agencies, and non-government organizations for assessing and monitoring genetic diversity.

Purpose of Laboratory

The purpose of the Laboratory is to analyze molecular genetic markers (protein and DNA) in plant material submitted by Forest Service employees and those from other cooperating entities. NFGEL provides baseline genetic information, determines the effect of management on the genetic resource, supports genetic improvement program, and contributes information in the support of conservation and restoration programs, especially those involving native and TES (threatened, endangered, and sensitive) species.

Alignment to National Strategic Plan for FY04-08

NFGEL's work aligns to the following National Strategic Plan measures:

1. Goal 1 (Reduce risks from catastrophic wildland fire)
2. Goal 2 (Reduce the impacts from invasive species).
3. Goal 4 (Help meet energy resource needs)
4. Goal 5 (Improve watershed condition)
5. Goal 6 (Mission related work in addition to that which supports the agency goals)

NFGEL Projects

NFGEL projects were processed to meet a variety of management objectives. Project results were used to guide restoration and conservation projects, and assist in silviculture and tree improvement activities. During FY 2004, NFGEL continued to follow its mission to "provide state-of-the-art molecular genetic information to the National Forests and other cooperating agencies for the evaluation and protection of our nation's genetic resource". Nine project reports are included in this Annual Report.

Valerie Hipkins
NFGEL Director
December 2004

Overview

NFGEL projects were processed to meet a variety of management objectives. Project results were used to guide restoration and conservation projects, and assist in silviculture and tree improvement activities. During FY 2004, NFGEL continued to follow its mission to "provide state-of-the-art molecular genetic information to the National Forests and other cooperating agencies for the evaluation and protection of our nation's genetic resource". Nine project reports follow.

Silviculture and Tree Improvement

1. **Genetic Diversity and Source ID of Monterey Pine (*Pinus radiata*) in Monterey County, CA**
   (NFGEL Project #98)
2. **Genetic Fingerprinting of Hybrid *Populus***
   (NFGEL Project #169)
3. **Ramet and Progeny Identification in a Port-Orford Cedar (Chamaecyparis lawsoniana) Disease Resistance Breeding Program**
   (NFGEL Project #183)
4. **Ploidy Variation in Acacia koa**
   (NFGEL Project #186)

Conservation and Restoration

1. **DNA Fingerprinting the 'George Washington Trees'**
   (NFGEL Project #129)
2. **Genetic Affinities of a Wallflower (*Erysimum)* Population from Lake Earl Dunes in Coastal Northern California**
   (NFGEL Project #144)
3. **Isozyme Analysis of *Arnica lessingii***
   (NFGEL Project #160)
4. **Genetic Variation in *Lupinus constancei:* Implications for Seed Transfer between Colonies**
   (NFGEL Project #163)
5. **Taxonomic identification of *Erythronium* (Fawn-lily) samples from the Olympic Peninsula, Washington**
   (NFGEL Project #170)

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Final Report

Genetic Diversity and Source ID of Monterey Pine (*Pinus radiata*) in Monterey County, CA

Brother Alfred Brousseau

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NFGEL Project #98

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November 23, 2004

MANAGEMENT SUMMARY

Objective 1: Did the planted Monterey Pine trees along Highway 1 in Monterey County near Carmel originate from one of the three natural mainland populations (Monterey, Cambria, or Año Nuevo)?

Population assignment tests indicate all three native populations may have contributed to the CalTrans plantings. It is entirely possible that the trees in each stand descended from more than one natural population, or from a population not analyzed in this study.

Objective 2: How genetically similar are the Hatton Canyon stands to other stands in the Monterey population?

The Hatton Canyon stands are very similar to other Monterey area stands based on this isozyme study. They can be considered part of the Monterey area population. Two unique alleles (not detected in other populations in this or previous studies) were detected in the Hatton Canyon stands.

Objective 3: Is the overall genetic variation reduced in selected pitch canker resistant material compared to the species as a whole, or compared to susceptible material? (Contingent on common garden material being available at a later date for genetic testing.)

As the required material was not provided to NFGEL, this objective was not addressed.

Objective 4: Is there evidence of genetic differentiation among stands occurring on different geomorphic surfaces in the Monterey population?

Although significant genetic differentiation is observed among stands within the whole Monterey population, no significant differentiation among stands occurring on different geomorphic surfaces was observed in the Monterey population.

Objective 5: Is there evidence of isolation by distance among the stands located in the Monterey population?

No evidence of isolation by distance was observed among stands within the Monterey population.

INTRODUCTION

Monterey Pine, *Pinus radiata* D. Don, occurs naturally in five small, discrete areas. The Año Nuevo, Cambria, and Monterey populations are located on the coast of southern California. Two populations occur on the Islands of Guadalupe and Cedros off the coast of Baja California (Millar *et al.* 1988). The species is now planted as an ornamental and for timber in appropriate habitats throughout the world. Previous isozyme studies (Millar *et al.* 1988; Moran *et al.* 1988; Plessas and Strauss 1986) and other studies (e.g. Cool and Zavarin 1992) have shown that (1) Monterey Pine has moderate to low levels of variation compared to other conifers, (2) the five populations are somewhat differentiated from each other, and (3) stands within each population are very similar to each other.

Proposed improvements to Highway 1 near Carmel will require removal of standing Monterey Pine trees in Hatton Canyon. Two issues complicate the plan to replace these trees when road construction is finished. First, replanted trees must be resistant to the pitch canker which infects many trees in Hatton Canyon. Second, the trees must be native to the area. The first concern will be addressed elsewhere. The second is the subject of this study.

The word "native" can be ambiguous. Any Monterey Pine could be said to native to the Hatton Canyon area because Monterey Pines have grown in that area for millennia. However, Monterey Pine exists in five differentiated populations. Trees from the Cambria and Año Nuevo populations are not truly native to the Hatton Canyon area. Only trees from natural Monterey area stands can be considered native to Hatton Canyon. Further, not all descendents of Monterey area pines can be considered native to that area now. Cultivated lineages of Monterey Pine become genetically uniform. Trees from a lineage that has been cultivated for several generations might change local population genetics as much as using trees from Cambria or Año Nuevo.

There are two reasons to use truly native trees for revegetation along Highway 1. First, native Monterey area trees may be better adapted to the Hatton Canyon area than trees from the Año Nuevo or Cambria populations. Second, introducing Año Nuevo or Cambria genes would change population genetics of local Monterey area trees. Conserving biodiversity by preserving native Monterey Pine genotypes is especially important because the native populations are reservoirs of genetic variation for improving cultivated Monterey Pines world-wide.

Additional information about the genetic structure of the Monterey population may provide insight in developing management and replanting strategies. For instance, seeding or replanting activities may have to consider the soil type at the source population and restoration site if genetic variation is found to vary with geomorphic surface. In addition, if gene flow is sufficiently limited between stands, resulting in isolation by distance, seed should only be moved a limited distance to maintain these patterns.

This study evaluates isozyme diversity to determine the origins of Monterey Pine stands planted along Highway 1, and describe the genetic structure of Hatton Canyon stands and the Monterey population as a whole. This information will be used to inform management activities including revegetating Highway 1 after construction. Specifically, this study will address four objectives. First, did the planted Monterey Pine trees along Highway 1 in Monterey County near Carmel originate from one of the three natural mainland populations (Monterey, Cambria, or Año Nuevo)? Second, how genetically similar are Hatton Canyon stands to other stands in the Monterey population? Third, is there evidence of

genetic differentiation among stands occurring on different geomorphic surfaces in the Monterey population? Fourth, is there evidence of isolation by distance among the stands located in the Monterey population? A final objective investigating whether overall genetic variation is reduced in selected pitch canker resistant material, which was contingent on common garden material being available, will not be addressed, as no such material was made available for testing.

METHODS

Study Species and Samples. Samples were provided as seed from 254 trees located in natural and planted Monterey Pine populations (Table 1). Collections included samples from eleven planted stands along Highway 1 in Monterey County (referred to as the CalTrans stands) and two Hatton Canyon stands. Most seed samples contained 30 seed/tree. Seeds from each tree were packaged individually, and were surface sterilized by soaking them in 20% bleach at UC Davis. Approximate distances between stands within the Monterey population were provided in km, as was the geomorphic soil type found at each site, for analysis of Objectives 4 and 5 (available upon request).

Tissue Preparation. Fifteen seeds per tree were soaked in 1% hydrogen peroxide for 48 hours, rinsed, and plated out in petri dishes lined with Kimpack germination paper soaked in 1% hydrogen peroxide. Each petri plate contained seed from a single tree. When the seed had just germinated (when the radical just emerged from the seed coat), the embryo tissue was removed and the megagametophyte was ground in 90 µl of 0.2M phosphate extraction buffer (USDA Forest Service 2000). Wicks (2.0 mm wide, made of Whatman 3MM chromatography paper) were soaked in the resulting slurry, and frozen at -70ºC pending electrophoresis.

Electrophoresis. Methods of sample preparation and electrophoresis follow the general methodology of Conkle *et al.* (1982), with some modifications (USDA Forest Service 2000). All enzymes were resolved on 11% starch gels. A lithium borate electrode buffer (pH 8.3) was used with a Tris citrate gel buffer (pH 8.3) (System LB; Conkle *et al.* 1982) to resolve alcohol dehydrogenase (ADH), leucine aminopeptidase (LAP), malic enzymes (ME), phosphoglucose isomerase (PGI), and phosphoglucomutase (PGM). A sodium borate electrode buffer (pH 8.0) was used with a Tris citrate gel buffer (pH 8.8) (System SB; Conkle *et al.* 1982) to resolve catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH), glutamate-oxaloacetate transaminase (GOT), glucose-6-phosphate dehydrogenase (G6PDH), triosephosphate isomerase (TPI), and uridine diphosphoglucose pyrophosphorylase (UGPP). A morpholine citrate electrode and gel buffer (pH 6.1) (System MC6; USDA Forest Service 2000) was used to resolve diaphorase (DIA), fluorescent esterase (FEST), fructose-1,6-diphosphatase (FDP), isocitrate dehydrogenase (IDH), phosphogluconate dehydrogenase (6PGD), and malate dehydrogenase (MDH), and shikimate dehydrogenase (SKD). Enzyme stain recipes follow USDA Forest Service (2000). Two loci were resolved for DIA, FDP, GOT, LAP, PGI, PGM, SKD, TPI, and UGPP, three loci for 6PGD, and four for MDH, for a total of 31 loci.

Two people independently scored each gel, and a third person resolved any disagreement between scores. For further quality control, 10% of the individuals were run and scored twice. Gels were photographed, and the photographs consulted to resolve quality

control issues. Genetic interpretations were inferred directly from isozyme phenotypes based on knowledge of the generally conserved enzyme substructure, compartmentalization, and isozyme number in higher plants (Gottlieb 1981, 1982; Weeden and Wendel 1989), and previous knowledge of Monterey Pine isozymes (e.g. Millar *et al.* 1988; Moran *et al.* 1988; Plessas and Strauss 1986).

Data Analysis. Six standard measures of genetic diversity were calculated from the final data set: the average number of samples per population scored for each locus (*N*), the percent polymorphic loci (*P*), the average number of alleles observed at each locus (*A*), the average number of alleles observed at each polymorphic locus (A_P), the observed heterozygosity (H_o), and the expected heterozygosity (H_e). A locus was considered polymorphic if an alternate allele occurred even once. Calculations were performed using AlleleFreq version 4.0 (a program by J. Nason).

Prior to running assignment tests, those loci displaying the greatest differentiation among source populations were identified using a Canonical Discriminant Analysis employed by SAS (SAS Institute, Inc.; performed by Bob Westfall, USDA Forest Service, PSW). Only those loci determined to be informative were used in further population assignment analyses.

In order to estimate the origin of the stands of Monterey pine planted along Highway 1 (Año Nuevo, Cambria, or Monterey), population assignment tests were completed for the CalTrans stands based on the isozyme data described above. These analyses assign an individual of unknown origin (in this study, CalTrans stands) to potential source populations (here, three native Monterey pine populations) based on the multilocus genotype of the individual and the allele frequencies observed in the source populations. For these analyses, loci and alleles in this study were matched to two previous studies of Monterey Pine isozymes (Millar *et al.* 1988, Plessas and Strauss 1986). From this combined data, the allele frequencies for each of the three native populations were calculated as the weighted average of the three data sets. That is, for all allele frequencies (*p)* for each of the *i* data sets, the weighted average allele frequency ($\overline{p}_w$) was calculated as: $\overline{p}_w = \frac{\sum_i n_i p_i}{\sum_i n_i}$. Hatton Canyon allele frequencies were included in the Monterey data set for the NFGEL data. In order to provide infiles in the proper format for the assignment analyses, these weighted average allele frequencies were used to create simulated populations of 1000 multilocus genotypes for each of the three native populations (Año Nuevo, Cambria, Monterey).

Two independent analyses were completed for the population assignment tests: the Bayesian likelihood algorithm employed by the program GeneClass (Cornuet *et al.* 1999), and the likelihood ratio tests employed by the program WhichRun (Banks and Eichert 2000). The Bayesian likelihood algorithm was employed using the program GeneClass for two types of assignment tests: the direct assignment test, which assigns each unknown individual to exactly one source population, and the simulation test, which assigns the individual to potentially multiple source populations, or none of the populations, based on its likelihood of arising in each. The program WhichRun estimates the "likelihood" of an unknown individual originating in a source population as the Hardy-Weinberg probability of its multilocus genotype occurring from the observed allele frequencies in that population. Two assignment tests were completed using this program: the direct assignment test, where each individual was assigned to exactly one source population, and the critical population LOD score, a more stringent test in that a sample is only assigned to a population (the critical population) if its

likelihood in that population is at least 10 times greater than the next likely population. Thus, a total of four assignment tests were performed for the CalTrans data.

In order to describe the genetic similarity of the Hattan Canyon stand to the other Monterey stands of Monterey pine, two standard measures of genetic structure were estimated: θ_P, a measure of population differentiation analogous to F_{ST}, and Lewis and Zaykin's (2001) coancestry identity, which is analogous to genetic distance. Significance of θ_P was estimated from 1000 bootstrap replicates over all loci. All isozyme loci were included in analyses of the Hatton Canyon population (as opposed to the subset of loci included in the assignment tests). All estimates of genetic variance were generated by the software GDA (Lewis and Zaykin 2001). Using the matrix of coancestry identity for all Monterey stands, a Neighbor-Joining phenogram was built, and its significance estimated over 1000 bootstrap replicates, using the program PHYLIP (Felsenstein 1993).

Two analyses were completed in order to determine whether the geomorphic substrate or geographic distance between stands better explains the genetic structure of the Monterey population. First, in order to determine if stands occurring on different geomorphic surfaces are genetically differentiated, stands were grouped into "regions" based on the soil substrate present at each site. The Jack's Peak stand was removed from this analyses due to assumptions of the analysis program which require at least two stands (or subpopulations) per soil type (population; Lewis and Zaykin 2001), and Jack's Peak was the only stand occurring on inland granitics. A hierarchical analysis of genetic variation was then completed for two models using the program GDA (Lewis and Zaykin 2001). The two-level hierarchical model does not distinguish between soil types, and estimates genetic differentiation among individuals within stands (f) and among stands within the entire population (θ_P). The three-level hierarchical model estimates genetic differentiation among individuals within stands (f), among stands occurring on the same soil (θ_S) and among stands occurring on the same soil-type within the entire population (θ_R). The null hypothesis of no genetic differentiation among stands occurring on different soil types was rejected only if genetic differentiation among soil-types at the three-level hierarchy (θ_R) was significantly different from zero. Significance of measures of allele-frequency variance were determined from 95% confidence intervals estimated by bootstrapping over 1000 replicates.

Second, Slatkin's (1993) test for isolation by distance was used to determine if genetic similarity between stands decreases as a function of geographic distance among stands in the Monterey population. Slatkin (1993) showed that $\widehat{M}$ (an estimator of N*m*) is inversely proportional to geographic distance between stands on a logarithmic scale, with the slope of this relationship varying with the type of dispersal occurring in the species (slope of -1 indicative of a one-dimensional stepping stone model, and a slope of –1/2 indicative of a two-dimensional stepping stone model). For each pair of stands, $\widehat{M}$ was calculated from the pairwise F_{ST} (estimated using J. Nason's program AlleleFreq) using the equation: $\widehat{M} = \frac{1}{4}(\frac{1}{F_{ST}} - 1)$. The log of each $\widehat{M}$ was regressed against the log geographic distance for each pair of stands, and the correlation characterized using Pearson's r.

RESULTS

Sampling. The viability of seed provided for this study sometimes was low. Seed from 13 trees failed to germinate and low germination rates were noted in other trees (data available upon request). The most seriously affected stands were Cambria, both Hatton Creek stands, and CalTrans stands 1, 2, 3, and 7, with poor seed quality reducing the sample size from the planned ten to the equivalent of 6 to 8.5 trees per stand.

Genetic Diversity. Levels of genetic variation observed in the three native populations of Monterey pine (Año Nuevo, Cambria, and Monterey) were lower than those reported in previous studies (Millar *et al.* 1988; Moran *et al.* 1988; Plessas and Strauss 1986), likely due to small sample sizes in this study. Based on approximately 10 samples for each of the Año Nuevo and Cambria populations, and 120 for the Monterey population, levels of genetic diversity ranged from 16.1–67.7 percent polymorphic loci, 1.2-2.4 alleles per locus, and fixation indices from –0.024 - 0.085 (Table 2). The Monterey population contained greater levels of allelic diversity than the other populations (Monterey = 2.39 alleles per locus, Año Nuevo = 1.55, Cambria = 1.23). Cambria contained lower levels of observed heterozygosity that the other populations (Monterey = 0.095, Año Nuevo = 0.110, Cambria = 0.049). Although Año Nuevo contained slightly more variation than Cambria, and several alternate alleles were observed in higher frequencies in Año Nuevo than the other native populations (Appendix), no unique alleles were observed in this population in this study. One unique allele that was observed in the Cambria population was also observed in two CalTrans stands (stands 6 and 7). This allele, MDH4-2, was also reported in Cambria by Millar *et al.* (1988).

CalTrans stands contained higher levels of polymorphism than any of the native populations (70.97 percent polymorphic loci), and had levels of allelic diversity comparable to the Monterey population (2.23 alleles per locus, observed heterozygosity 0.11). Interestingly, three alleles were observed in the CalTrans stands that were not observed in the native stands in this study: 6PGD2-2, GOT1-3, and FDP1-2. Allele 6PGD2-2 was previously reported in all three native stands by Millar *et al.* (1988) and by Plessas and Strauss (1986), and allele GOT1-3 was previously reported in the Monterey population by Millar *et al.* (1988). However, allele FDP1-2 was not observed by either of the pervious studies and appears unique to CalTrans stands 2, 7, 9, and 10.

Source Identification. Canonical Discriminant Analysis based on data simulated from the weighted allele frequencies identified six informative loci, which were included in the population assignment tests: 6PGD2, ADH, GOT3, LAP2, MDH4, and UGPP2. Admixture analyses indicate that all native populations are potential sources for trees sampled from CalTrans stands. Direct assignment using the Bayesian logarithm employed by GeneClass indicate that 63% of the samples are from Monterey, 25% from Año Nuevo, and 12% from Cambria (Figure 1A). Direct assignment tests using the Hardy-Weinberg likelihoods employed by WhichRun were similar, and indicate that 55% of samples are from Monterey, 33% from Año Nuevo, and 12% from Cambria (Figure 2). WhichRun LOD scores identified 14 individuals to be at least 10 times more likely to be assigned to the critical population than the next likely score: four (4%) to Cambria and ten (9%) to Año Nuevo. Simulation estimates using the Bayesian likelihood logarithm employed by GeneClass, which take into account the genetic similarity of native populations and thus the possibility of assigning an individual to more than one source, assigned 66% of CalTrans samples to all

three native populations, 28% to both Año Nuevo and Monterey, 4% to Cambria, 1% to Monterey, and 1% to none of the sources (Figure 1B).

Power analyses were completed using a simulated population of 1000 individuals for each native population in order to describe the ability of this data set to distinguish between native populations. Power analyses for the direct assignment test using the Bayesian logarithm correctly assigned 73% of Monterey samples, 64% of Cambria samples, and 86% of Año Nuevo samples to the simulated source population. Power analyses for the simulation tests using the Bayesian logarithm assigned 82% of Monterey individuals to all three native populations, and correctly assigned 2% to Monterey alone; 51% of Cambria individuals to all three native populations, and 27% to Cambria alone; 54% of Año Nuevo individuals to both Año Nuevo and Monterey, and 28% to Año Nuevo alone.

Hatton Canyon Diversity. The two Hatton Canyon stands (HC1, HC2) analyzed in this study contained levels of genetic variation consistent with those observed at other stands within the Monterey population (P = 41.94, 38.71; A = 1.68, 1.58; H_o = 0.11, 0.07). Two unique alleles were observed in the Hatton Canyon stands: IDH-3 in HC1, and G6PD2-3 in HC2. Neither allele was observed by Millar *et al.* (1988) or Plessas and Strauss (1986) in any native stand. The population phenogram based on coancestry identity using Neighbor-Joining methods, while not highly supported based on bootstrap analyses, indicates that the Hatton Canyon stands are nested within the Monterey population, and are not unique or outliers from the other stands.

Population Differentiation. No significant support was found for the three-level hierarchical model testing genetic differentiation among stands on different soil types. Both the two-level and the three-level models resulted in non-significant fixation indices (f = -0.080, P>0.05 for both models). Although significant genetic differentiation among stands within the entire Monterey population was observed (θ_P = 0.077, P<0.05), and among stands within soil types (θ_S = 0.083, P<0.05), no significant differentiation among soil types was observed (θ_R = 0.019, P>0.05).

No evidence of isolation by distance was observed among stands within the Monterey population. The regression of log $\widehat{M}$ against log geographic distance resulted in a slope of 0.061 (R^2=0.0007), and r = 0.027.

DISCUSSION

Genetic Diversity. The small sample size of two of the native populations examined in this study (Año Nuevo and Cambria) makes direct comparison of the genetic diversity among populations difficult for this data set. However, the levels and patterns of genetic diversity observed among populations in this study are consistent with previous studies (Millar *et al.* 1988; Moran *et al.* 1988; Plessas and Strauss 1986) where we were able to match 18 loci among studies. Genetic variation, as measured by mean alleles per locus and percent polymorphic loci, were observed to be greater in the Monterey population than either of the other native populations, which is consistent with the previous studies. However, the lower levels of genetic diversity observed in the Cambria population is inconsistent with the previous studies, and is likely an artifact of the small sample size included here. Allele frequencies at 6PGD2, ADH, GOT3, LAP2, MDH4, and UGPP2 were particularly important for distinguishing among native Monterey Pine populations. Some of these same loci were

important in previous studies (6PGD, ADH, LAP, Millar *et al.* 1988; ADH, LAP, Moran *et al.* 1988; 6PGD, ADH, GOT, Plessas and Strauss 1986).

Source Identification. Based on the results of the various assignment tests, the trees planted along Highway 1 near Carmel ("CalTrans" trees) likely did not originate from a single source. The high levels of genetic diversity (mean alleles per locus = 2.2, percent polymorphic loci = 71.0) and heterozygosity expected under Hardy-Weinberg equilibrium (H_E = 0.110) observed among the CalTrans stands are larger than that observed in any native population in previous studies (Table 2, Millar *et al.* 1988; Moran *et al.* 1988; Plessas and Strauss 1986). Such elevated levels of diversity can result from the admixture of multiple sources (here, native populations) of trees. Indeed, population assignment tests indicate multiple origins for the CalTrans trees (Figures 1 and 2). The direct assignment tests consistently assign the majority of trees to the Monterey population (55 - 63%), although the more stringent LOD scores analysis uniquely assigned trees only to the Cambria (n = 4) and Año Nuevo (n = 10) populations. Further, the more informative simulation test using the Bayesian likelihood algorithm assigned the majority of CalTrans trees to all three native populations (66%), a quarter of the samples to both Año Nuevo and Monterey (28%), and only a fraction to Cambria (4%) and Monterey (1%) alone.

Thus, it appears that the direct analyses assign the majority of trees to the Monterey population, while the more stringent LOD scores and simulation tests assign only a few. Two factors may explain these seemingly conflicting results. First, power analyses reveal that the genetic similarity between the native populations in this data set reduces the ability of the tests to correctly assign "known" individuals. Notably, the simulation tests assigned more than 50% of individuals to all three populations, regardless of native stand of origin. A similar amount of error must be expected for the CalTrans trees. Second, the native stands used in this study (Año Nuevo, Cambria, and Monterey) are not an exhaustive sampling of potential sources of the CalTrans trees. Two additional native populations (Guadalupe Island and Cedros Island) were not included in this study, and as such, the likelihood that CalTrans trees may have originated in either population cannot be addressed. Similarly, New Zealand has an important breeding program for Monterey pine, and it is possible that trees in the CalTrans stands may have been reintroduced from these populations. If this were the case, it is likely the genetic structure of the New Zealand trees would differ from those observed in the native stands in California, even if the original breeding stock were from those native stands, due to genetic drift or selection. The chance that a true source population was not analyzed in this study is the most likely explanation for the observation of a unique allele observed in the CalTrans stands (FDP1-2; Appendix). Again, the likelihood that CalTrans trees may have originated in an unsampled native stand or New Zealand cannot be addressed since samples from these populations were not included in this analysis.

Hatton Canyon Diversity. The Hatton Canyon stands analyzed in this study do not appear genetically unique when compared to the other stands in the Monterey population. Levels of genetic diversity and heterozygosity observed in the Hatton Canyon stands were consistent with those observed in the other Monterey stands (Table 3). Two private alleles were found in these stands (G6PD2-3 and IDH-3), but other alleles were found to be unique to other stands in this population (*e.g.* GOT3-3 in PC, FDP2-2 in MT3, IDH-5 in DR, and PGI2-4 in JP). Based on these data, any designation of these Hatton Canyon stands as genetically unique, especially if based on the occurrence of a single private allele in each stand, is weak. Management activities designed around this information (reseeding, outplanting, or other

efforts) should also consider the private alleles found in other stands within the Monterey population.

Population Differentiation. Results of the hierarchical analyses of genetic differentiation based on geomorphic surface data indicate that soil type is not correlated with genetic differentiation among stands within the Monterey population. Significant genetic differentiation found among stands within the Monterey population is consistent with the observation of private alleles identified in several stands, but no pattern was found among soil types. This result indicates that those processes influencing genetic structure in Monterey pine is not limited (*e.g.* breeding and dispersal) by the geomorphic surface within the Monterey population. The levels of genetic differentiation observed among stands in this study (θ_P = 0.077) were larger than those reported in previous studies (1.3-2.0% diversity among stands, Moran *et al.* 1988), and may be a consequence of the small number of trees sampled from each stand.

The lack of significance in the correlation analysis for isolation by distance indicates that gene flow does not decrease as a function of distance in the Monterey population. This result is consistent with previous studies, which found that gene flow is not restricted within populations (Moran *et al.* 1988) and the general theory that gene flow, occurring primarily through pollen dispersal, routinely takes place over long distances in gymnosperms (Hamrick and Nason 2000). Neither the geomorphic surface hypothesis nor the isolation by distance hypothesis sufficiently explains the genetic structure observed in the Monterey population, and together these analyses indicate that other factors, including random events, may better explain the genetic structure in this species.

SUMMARY

Monterey pine planted along Highway 1 by CalTrans likely originated from all three native stands (Año Nuevo, Cambria, and Monterey), and may have originated from other sources not analyzed in this study. The relatively high levels of variation observed in the CalTrans stands are consistent with the hypothesis that the trees originated in more than one source population. The Hatton Canyon stands, while containing two private alleles, contain similar levels of genetic variation and heterozygosity as the other stands within the Monterey population, and should not be considered unique. No evidence was found that genetic differentiation is influenced by geomorphic surface type or isolation by distance among stands within the Monterey population of Monterey pine.

LITERATURE CITED

Banks, M. A. and W. Eichert. 2000. WHICHRUN (Version 3.2) a computer program for population assignment of individuals based o multilocus genotype data. Journal of Heredity 91: 87-89.

Conkle, M. T., Hodgskiss, P. D., Nunnally, L. B., and Hunter, S. C. 1982. Starch Gel Electrophoresis of Conifer Seeds: a Laboratory Manual. Gen. Tech. Rep. PSW-64. Berkeley, CA: Pacific Southwest Forest and Range Experiment Station, Forest Service, U.S. Department of Agriculture. 18pp.

Cool, L. G. and E. Zavarin. 1992. Terpene variability of mainland *Pinus radiata.* Biochemical systematics and Ecology 20: 133-144.

Cornuet, J. M., S. Piry, G. Luikart, A. Estoup, and M. Solignac. 1999. New methods employing multilocus genotypes to select or exclude populations as origins of individuals. Genetics 153(4): 1989-2000.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. *Distributed by the author. Department of Genetics, University of Washington, Seattle.*

Gottlieb, L. D. 1981. Electrophoretic evidence and plant populations. Prog. Phytochem. 7: 1-46.

Gottlieb, L. D. 1982. Conservation and duplication of isozymes in plants. Science 216: 373-380.

Hamrick, J. L., and J. D. Nason. 2000. Gene flow in forest trees, pp. 81-90 *in* Young, A., D. Boshier, and T. Boyle, eds.. Forest Conservation Genetics, Principles and Practice. CSIRO Publishing, Collingwood, Australia, and CABI Publishing, Oxon, United Kingdom.

Lewis, P. O., and Zaykin, D. 2001. Genetic Data Analysis: Computer program for the analysis of allelic data. Version 1.0 (d16c). Free program distributed by the authors over the internet from http://lewis.eeb.uconn.edu/lewishome/software.html.

Millar, C. I., S. H. Strauss, M. T. Conkle, and R. D. Westfall. 1988. Allozyme differentiation and biosystematics of the California closed-cone pines. Systematic Botany 13: 351-370.

Moran, G. F., J. C. Bell, and K. G. Eldridge. 1988. The genetic structure and the conservation of the five natural populations of *Pinus radiata*. Can. J. For. Res. 18: 506-514.

Plessas, M. E., and S. H. Strauss. 1986. allozyme differentiation among populations, stands, and cohorts in Monterey Pine. Can. J. For. Res. 16: 1155-1164.

Slatkin, M. 1993. Isolation by distance in equilibrium and non-equilibrium populations. Evolution 47(1): 264-279.

USDA Forest Service. 2000. National Forest Genetic Electrophoresis Laboratory Standard Operating Procedures. NFGEL, USDA Forest Service, Camino, California.

Weeden, N. F. and Wendel., J. F. 1989. Genetics of plant isozymes. pp. 46-72 *in* Isozymes in Plant Biology, eds. D. E. Soltis and P. S. Soltis. Dioscorides Press, Portland, Oregon.

Table 1. Name and number of Monterey Pine samples submitted for isozyme analysis. No. Stands = the number of stands sampled from each population. Total No. Trees = the total number of trees sampled from each population.

Population	**No. Stands**	**Total No. Trees**
Año Nuevo	1	10
Cambria	1	10
Monterey (+HC)	12	126
(Hatton Canyon)	(2)	(26)
CalTrans	11	108
TOTAL	25	254

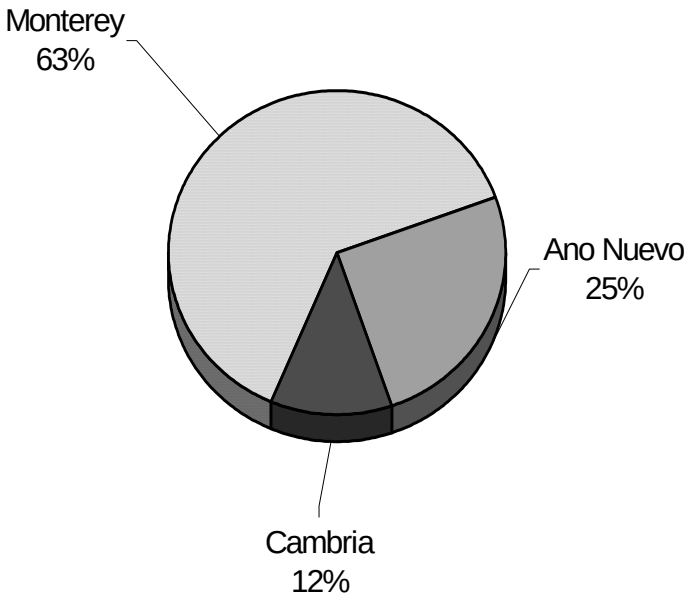
Table 2. Average genetic diversity per population of Monterey Pine. Estimates for the Monterey population includes data from two Hatton Canyon stands. Diversity measures for individual CalTrans stands are available upon request. N = average number of samples per locus. P = percent polymorphic loci. A = average number of alleles per locus. A_P = mean alleles per polymorphic locus. H_O = observed heterozygosity. H_E = expected heterozygosy. F = fixation index. Standard errors in parentheses.

Population	N	P	A	A_P	H_O	H_E	F
Entire Study	295.90	77.4	2.5 (0.2)	3.0	0.093 (0.028)	0.102 (0.029)	0.080 (0.028)
Año Nuevo	9.97	38.7	1.5 (0.1)	2.4	0.110 (0.029)	0.108 (0.029)	-0.024 (0.019)
Cambria	7.74	16.1	1.2 (0.1)	2.4	0.049 (0.025)	0.062 (0.029)	0.036 (0.037)
Monterey	118.77	67.7	2.4 (0.2)	3.0	0.095 (0.028)	0.105 (0.030)	0.085 (0.029)
CalTrans Stands	103.87	71.0	2.2 (0.2)	2.7	0.105 (0.028)	0.110 (0.029)	0.045 (0.034)

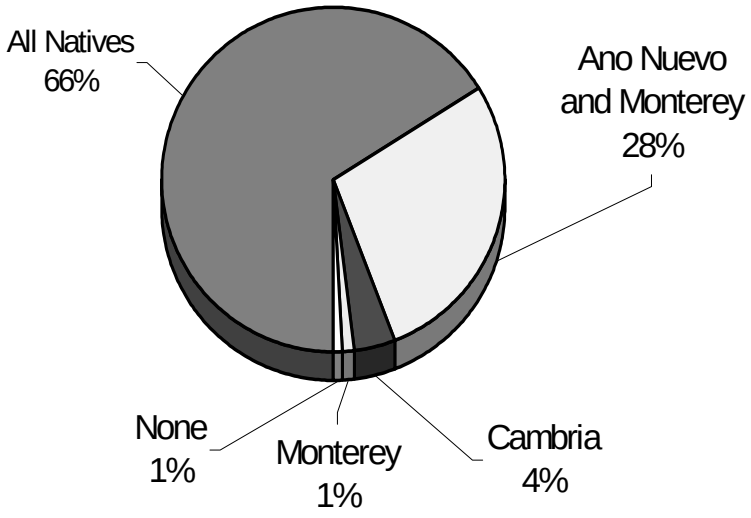
Table 3. Average genetic variation per stand of Monterey Pine within the Monterey population. Entire Population is the mean estimates over all stands. N = average number of samples per locus. P = percent polymorphic loci. A = mean alleles per locus. A_P = mean alleles per polymorphic locus. H_O = observed heterozygosity. H_E = expected heterozygosy. F = fixation index. Standard errors in parentheses.

Stand	**Abbr.**	***N***	***P***	***A***	A_P	H_O	H_E	***F***
Entire Population		118.8	67.7	2.4 (0.2)	3.0	0.095 (0.283)	0.105 (0.030)	0.085 (0.029)
Marine Terrace 2	MT2	9.8	25.8	1.4 (0.1)	2.4	0.061 (0.025)	0.086 (0.032)	0.056 (0.035)
Marine Terrace 3	MT3	9.9	45.2	1.6 (0.2)	2.4	0.094 (0.026)	0.113 (0.031)	0.034 (0.044)
Marine Terrace 5	MT5	9.9	38.7	1.5 (0.1)	2.3	0.087 (0.026)	0.099 (0.029)	0.025 (0.026)
Marine Terrace 6	MT6	9.7	29.0	1.4 (0.1)	2.4	0.097 (0.036)	0.097 (0.034)	-0.001 (0.019)
Youngest Sand Dunes	YSD	9.8	41.9	1.6 (0.1)	2.4	0.100 (0.031)	0.101 (0.032)	-0.015 (0.012)
Middle-Aged Sand Dunes	MSD	10.0	32.3	1.5 (0.2)	2.6	0.100 (0.037)	0.087 (0.030)	-0.030 (0.016)
Jack's Peak	JP	9.9	35.5	1.5 (0.2)	2.5	0.107 (0.034)	0.098 (0.032)	-0.031 (0.004)
Lobos Ranch	LR	9.7	35.5	1.5 (0.1)	2.4	0.084 (0.028)	0.095 (0.030)	0.038 (0.037)
Druid's Ranch	DR	9.7	45.2	1.6 (0.1)	2.3	0.135 (0.034)	0.116 (0.029)	-0.063 (0.019)
Pescadero Canyon	PC	9.8	35.5	1.4 (0.1)	2.2	0.094 (0.033)	0.089 (0.028)	-0.009 (0.026)
Hatton Canyon 1	HC1	11.7	41.9	1.7 (0.2)	2.6	0.110 (0.037)	0.120 (0.033)	0.076 (0.055)
Hatton Canyon 2	HC2	8.7	38.7	1.6 (0.2)	2.5	0.069 (0.021)	0.102 (0.028)	0.113 (0.052)

Figure 1. Proportion of trees sampled from CalTrans stands assigned to three native Monterey Pine populations using the Bayesian likelihood algorithm employed by the program GeneClass. A) Direct assignment of individuals to exactly one population. B) Simulation assignment of individuals to any population where the likelihood is greater than a critical value. See Methods for details.



A. Direct assignment.



B. Simulation assignment.

Figure 2. Distribution of trees sampled from CalTrans stands assigned to three native Monterey Pine populations using the direct assignment test employed by the program WhichRun. See Methods for details.

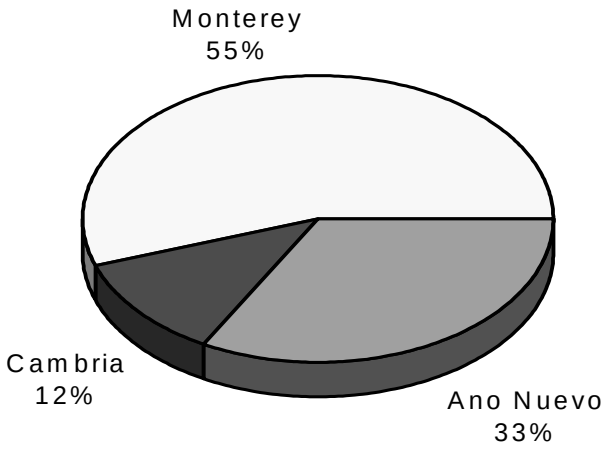
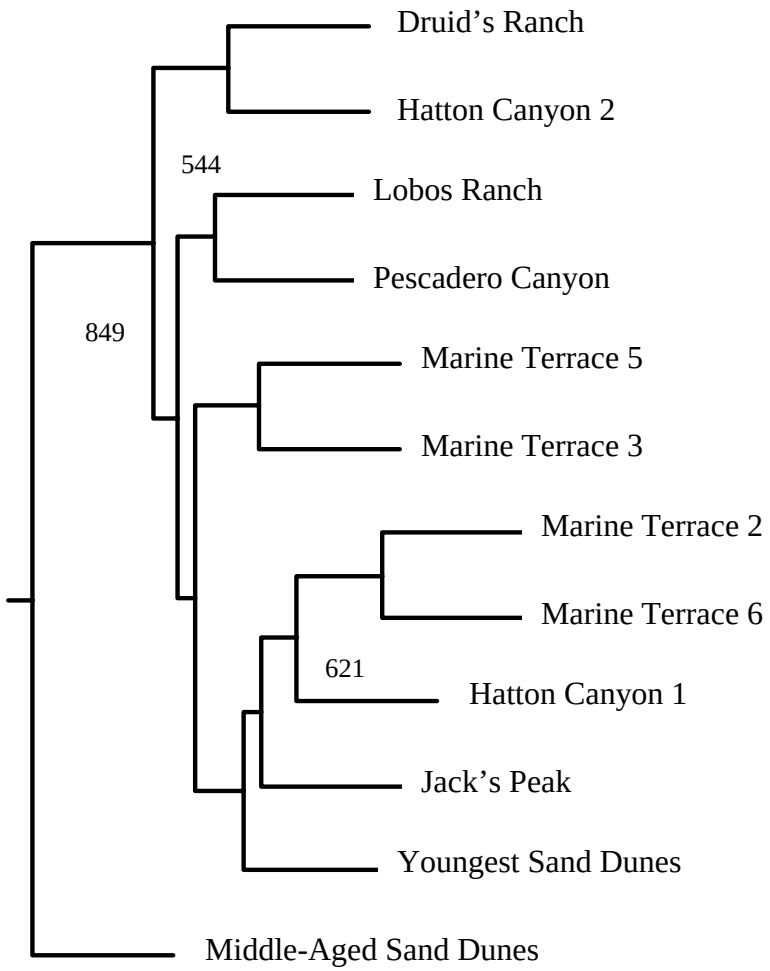


Figure 3. Neighbor-Joining phenogram of twelve stands sampled from the Monterey population of Monterey Pine. Bootstrap support over 1000 replicates indicated for nodes occurring in more than 50% of replicates.



Appendix. Allele frequencies observed in three native stands of Monterey Pine (Monterey, Año Nuevo, and Cambria), and those observed in CalTrans stands planted along Highway 101 in Monterey, CA. Allele frequencies by stand are available upon request. Migration is the distance in mm that the allele migrates from the origin.

Locus-Allele	Migration	Entire Study	Monterey	Año Nuevo	Cambria	CalTrans
6PGD1–1	24, 26	1.000	1.000	1.000	1.000	1.000
6PGD2-1	20	0.998	1.000	1.000	1.000	0.995
6PGD2-2	null	0.002				0.005
6PGD3-1	16	0.976	0.983	1.000	1.000	0.966
6PGD3-2	14	0.024	0.018			0.034
GOT1-1	29	0.989	0.975	0.900	1.000	0.986
GOT1-2	39	0.010	0.025	0.100		0.010
GOT1-3	35	0.001				0.005
GOT3-1	-9/6/17	0.959	0.984	0.900	1.000	0.933
GOT3-2	-6/7/18	0.039	0.012	0.100		0.067
GOT3-3	0/13/22	0.002	0.004			
ADH-1	14/15	0.461	0.483	0.600	0.444	0.423
ADH-2	11	0.329	0.308	0.150	0.444	0.361
ADH-3	null	0.021	0.038	0.050		
ADH-4	20	0.189	0.171	0.200	0.111	0.216
CAT-1	7	1.000	1.000	1.000	1.000	1.000
DIA1-1	23	0.993	0.985	1.000	1.000	1.000
DIA1-2	21	0.007	0.015			
DIA2-1	18	0.977	0.971	1.000	1.000	0.981
DIA2-2	21	0.023	0.029			0.019
FDP1-1	19	0.988	1.000	1.000	1.000	0.975
FDP1-2	16	0.012				0.026
FDP2-1	8	0.998	0.996	1.000	1.000	1.000
FDP2-2	11	0.002	0.004			
FEST-1	8	0.994	0.991	1.000	1.000	0.995
FEST-2	10	0.006	0.009			0.005
G6PD2-1	14	0.992	0.992	1.000	1.000	0.990
G6PD2-2	16	0.006	0.004			0.010
G6PD2-3	13	0.002	0.004			
IDH-1	16	0.975	0.946	1.000	1.000	0.943
IDH-2	19	0.003	0.008			0.005
IDH-3	22	0.001	0.004			
IDH-4	12	0.020	0.037			0.052
IDH-5	9	0.001	0.004			
LAP1-1	51	0.657	0.595	0.800	0.714	0.713
LAP1-2	52	0.312	0.372	0.150	0.286	0.257
LAP1-3	50	0.025	0.029	0.050		0.020
LAP1-4	null	0.006	0.004			0.010
LAP2-1	35	1.000	1.000	1.000	1.000	1.000
MDH1-1	22	1.000	1.000	1.000	1.000	1.000
MDH2-1	16	1.000	1.000	1.000	1.000	1.000
MDH3-1	6	0.970	0.946	0.850	1.000	0.933
MDH3-2	11	0.002	0.004			0.005
MDH3-3	7	0.019	0.025	0.150		0.048
MDH3-4	null	0.009	0.025			0.014
MDH4-1	2.5	0.990	1.000	1.000	0.938	0.981
MDH4-2	5	0.010			0.063	0.019
ME-1	23	0.932	0.917	0.950	1.000	0.942

Locus-Allele	Migration	Entire Study	Monterey	Año Nuevo	Cambria	CalTrans
ME-2	21	0.068	0.083	0.050		0.058
PGI1-1	40	1.000	1.000	1.000	1.000	1.000
PGI2-1	28.5	0.648	0.624	0.800	0.500	0.673
PGI2-2	22	0.156	0.178	0.100	0.063	0.144
PGI2-3	30	0.191	0.190	0.100	0.438	0.183
PGI2-4	33	0.004	0.008			
PGM1-1	44	0.990	0.984	1.000	1.000	0.995
PGM1-2	40	0.010	0.017			0.005
PGM2-1	28	0.874	0.897	0.800	0.857	0.856
PGM2-2	22	0.070	0.079		0.143	0.063
PGM2-3	30	0.043	0.017	0.200		0.063
PGM2-5	25.5	0.012	0.008			0.019
SKD1-1	19	0.994	0.979	1.000	1.000	0.995
SKD1-2	16	0.004	0.013			0.005
SKD1-4	null	0.002	0.008			
SKD2-1	16	0.975	0.963	0.950	1.000	0.929
SKD2-2	14	0.012	0.021			0.033
SKD2-4	17	0.009	0.008	0.050		
SKD2-5	null	0.003	0.004			0.029
SKD2-6	9	0.001	0.004			0.010
TPI1-1	61	0.866	0.847	0.889	1.000	0.876
TPI1-2	59	0.134	0.153	0.111		0.124
TPI2-1	45	1.000	1.000	1.000	1.000	1.000
UGPP1-1	52	0.875	0.847	0.850	1.000	0.900
UGPP1-2	47	0.064	0.066	0.100		0.062
UGPP1-3	50	0.045	0.066			0.029
UGPP1-4	48.5	0.017	0.021	0.050		0.010
UGPP2-1	24	0.877	0.946	0.550	1.000	0.819
UGPP2-2	25	0.002				0.005
UGPP2-3	23	0.101	0.017	0.450		0.171
UGPP2-4	27.5	0.021	0.037			0.005

United States Department of Agriculture

Forest Service

National Forest Genetic Electrophoresis Laboratory (NFGEL)

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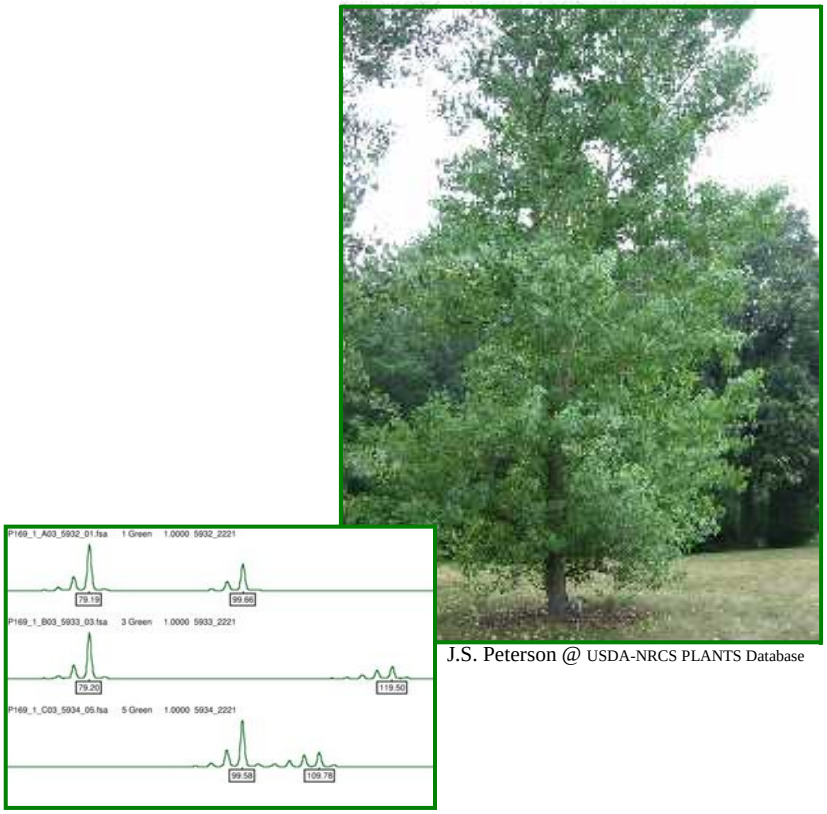
Final Report

Genetic Fingerprinting of Hybrid *Populus*



J.S. Peterson @ USDA-NRCS PLANTS Database

Report Prepared by: Robert C. Saich and Valerie D Hipkins
Contact: Valerie Hipkins, NFGEL Director, vhipkins@fs.fed.us
NFGEL Project #169

Submitted to: Private Company

September 14, 2004

PROJECT GOAL

To provide a 'genetic fingerprint' for seven hybrid poplar individuals.

MATERIALS

Samples of mature leaf tissue (3-5 leaves/individual) from seven *Populus* hybrid individuals were received on April 28, 2004. Leaf material was received from: (1) three *P. deltoides* X *P. trichocarpa* individuals, and (2) four individuals of *P. trichocarpa* X *P. nigra*.

METHODS

DNA Extraction. DNA extraction was carried out on leaf tissue using the DNeasy-96 Frozen Leaf Tissue Protocol following manufacturers instructions with tissue homogenization achieved via the Mixer Mill 300 (Qiagen). DNA quality and approximate quantity was assessed by visualizing all samples against 50ng of Lambda DNA standard on 0.8% agarose gels stained with EtBr under UV light.

SSR Amplification and Electrophoresis. Genetic fingerprints were created using a total of 12 SSR loci. Sources of SSR primers are provided in the prior NFGEL report: "Assessing SSR markers for paternity analysis in *Populus spp*", NFGEL Final Report, Project #162, February 18, 2004". SSR amplification and electrophoresis follow the protocols outlined in the NFGEL Project #162 final report. Briefly, 1.5 - 2.5 ng of template DNA was amplified in a 10 ul final volume including 1X PCR buffer, 2.0 mM $MgCl_2$, 0.4 uM of each dNTP, 0.4 uM of the forward and reverse primers, and 1 U of HotStarTaq DNA Polymerase (Qiagen). Amplifications were performed using a MJ Research PT-100 thermal controller following a touchdown protocol with annealing temperatures from 55°C to 50°C. Samples were denatured and loaded on an ABI Prism 3100 Genetic Analyzer for detection of SSR product. Samples were analyzed more than once to verify the observed patterns.

Analysis. ABI software packages, GeneScan® Analysis Software and Genotyper® Software v 3.7, were used to visualize and evaluate alleles at each locus. DNA fragment sizes calculated by the ABI software are reported here without adjustments.

RESULTS AND DISCUSSION

The SSR markers were successful at uniquely identifying the seven hybrid poplar individuals. Therefore, each of the seven trees has a unique "genetic fingerprint".

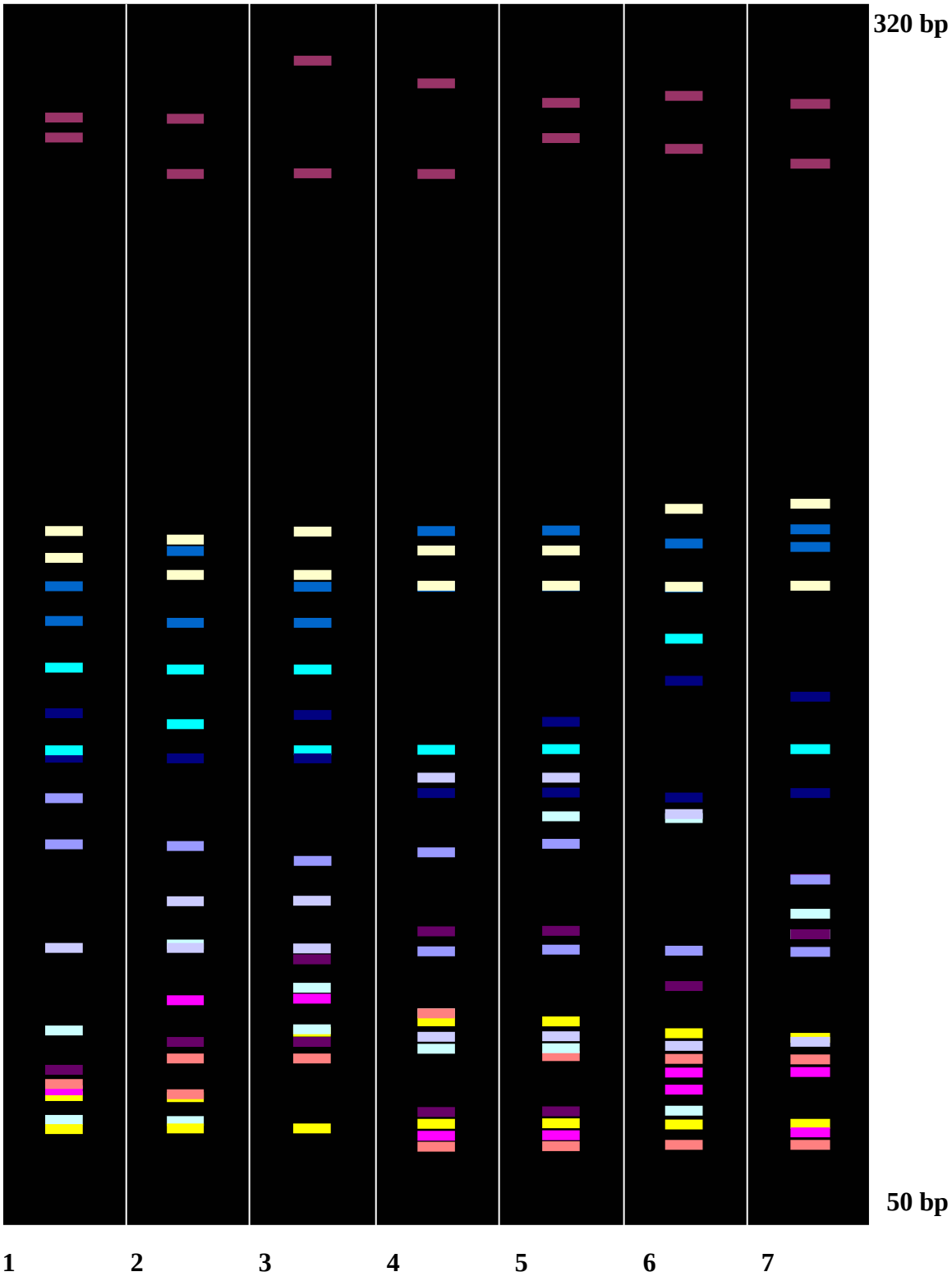
Three of the 12 SSR markers (markers 2885, 2235, and 2221) individually provide unique patterns. These seven trees can, therefore, be identified from each other by simply running only one of these three markers.

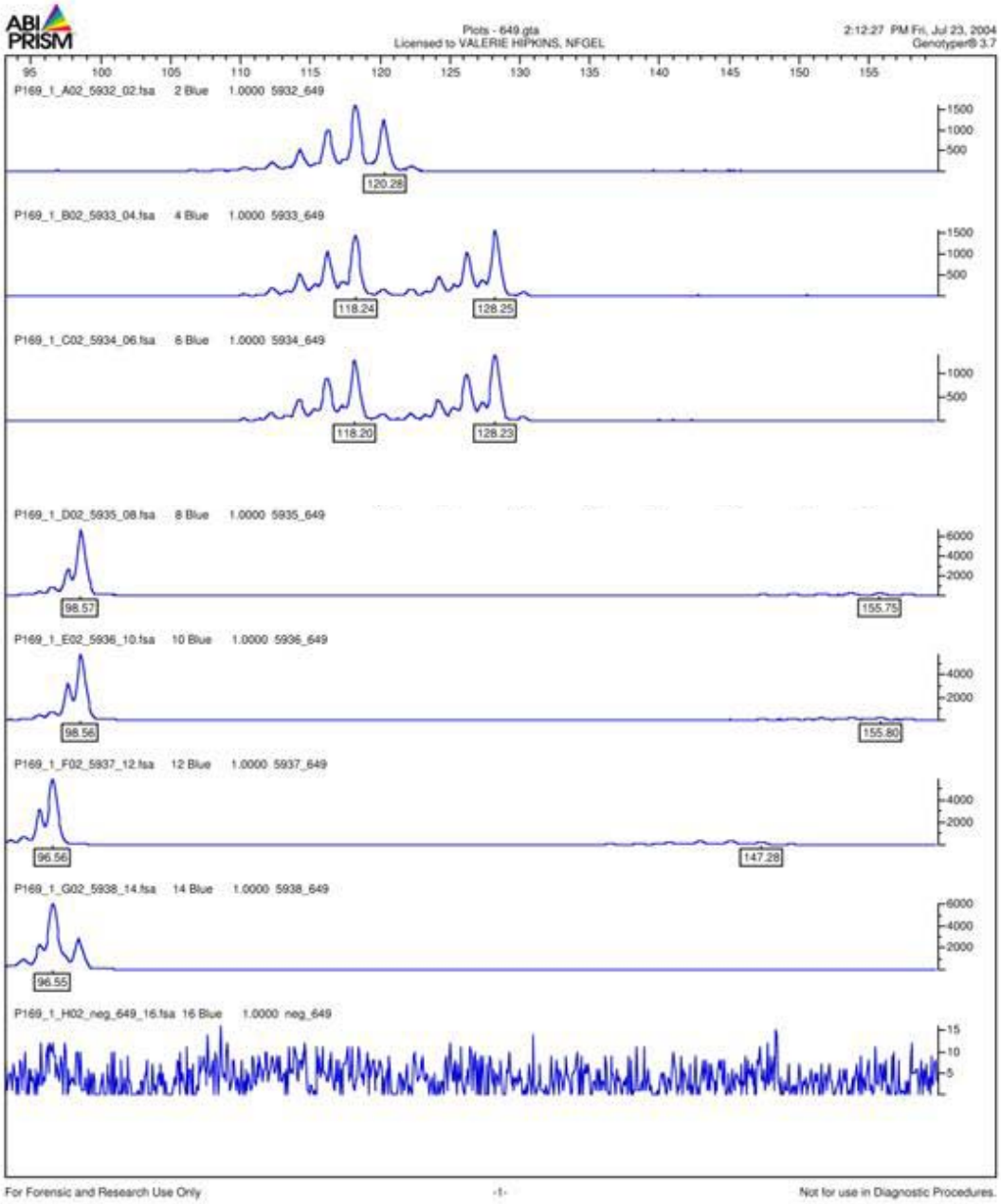
The genetic fingerprint data for all 12 SSR markers are presented in three different formats: (1) as a table (TABLE, pg 3) with SSR fragments per individual tree indicated by their base pair size, (2) as a graphical representation (pg 4) of the data found in the table, and (3) the ABI output of trace files per SSR marker (pgs 5 – 16). All three formats show the same data, just represented in different formats. The graphical representation of the data (pg 4) can be used to visualize the fingerprints of all the trees at all the loci assayed. A unique pattern is observed for each tree. These markers can be used to identify these individual trees in the future.

TABLE: Genotype data for seven *Populus* hybrid individuals at 12 SSR loci. Values are in basepairs.

SSR Locus	**INDIVIDUAL**						
	P. deltoides* X *P. trichocarpa			***P. trichocarpa* X *P. nigra***			
	1	**2**	**3**	**4**	**5**	**6**	**7**
649	120	118	118	99	99	97	97
	120	128	128	156	156	147	97
576	163	168	163	163	163	188	163
	181	181	181	163	163	188	163
433	206	202	202	198	198	198	198
	212	210	212	208	208	217	217
420	87	85	93	72	72	72	72
	87	93	93	103	93	93	93
2885	298	291	291	291	299	295	293
	304	304	316	311	307	309	307
2804	90	96	96	81	81	109	121
	90	96	115	121	121	109	133
2675	161	161	161	152	152	152	152
	171	161	171	152	168	177	173
2571	77	77	77	78	78	78	78
	84	84	99	101	101	99	97
2235	142	142	137	117	117	117	117
	150	142	137	139	142	117	133
2221	79	79	100	96	96	81	126
	100	119	110	104	146	146	126
2011	87	107	107	76	76	87	76
	87	107	107	76	76	91	91
14	192	192	192	200	200	200	208
	200	208	200	212	212	209	212

Graphical representation of DNA fingerprint data for seven *Populus* hybrid individuals at 12 SSR markers.





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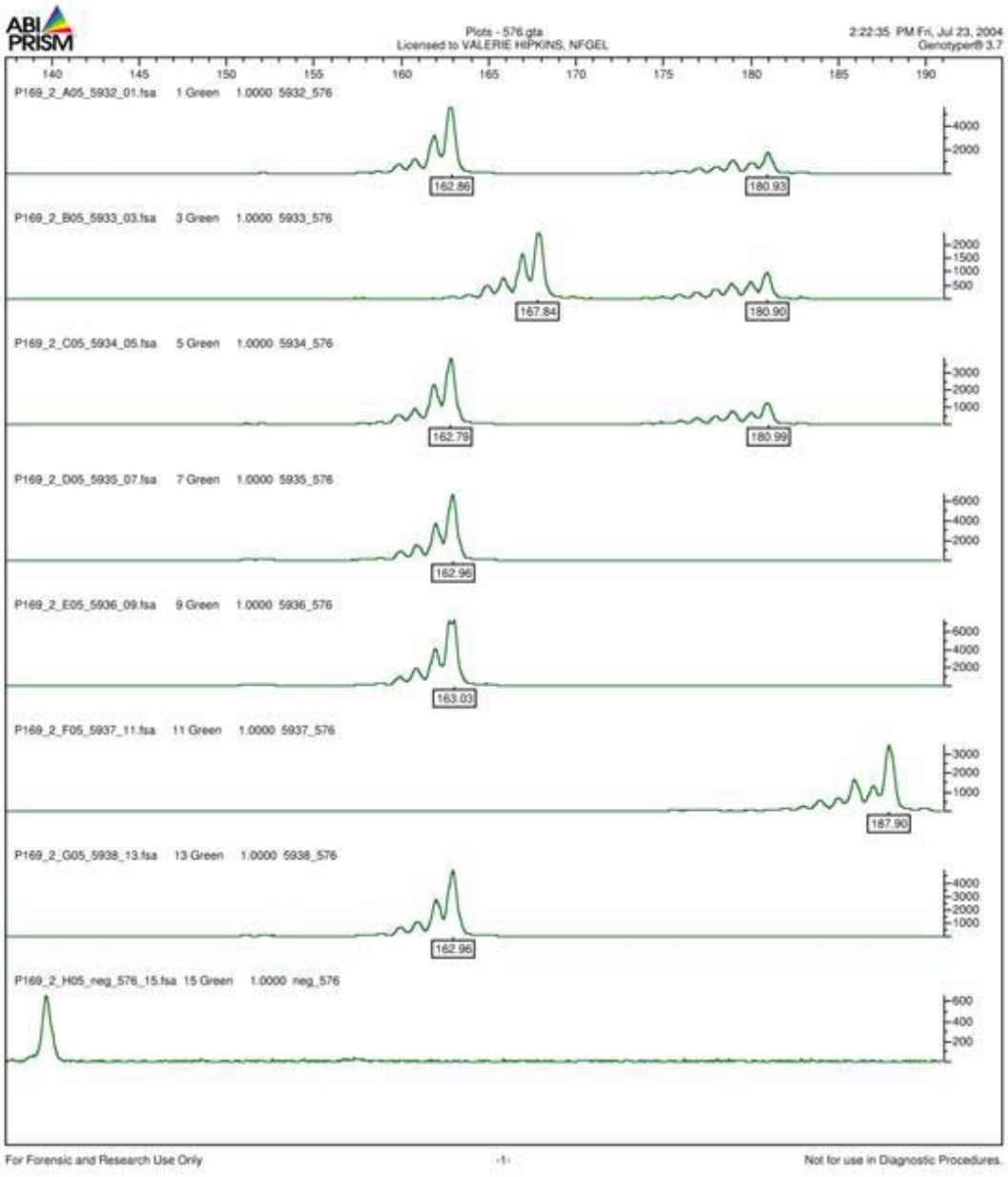

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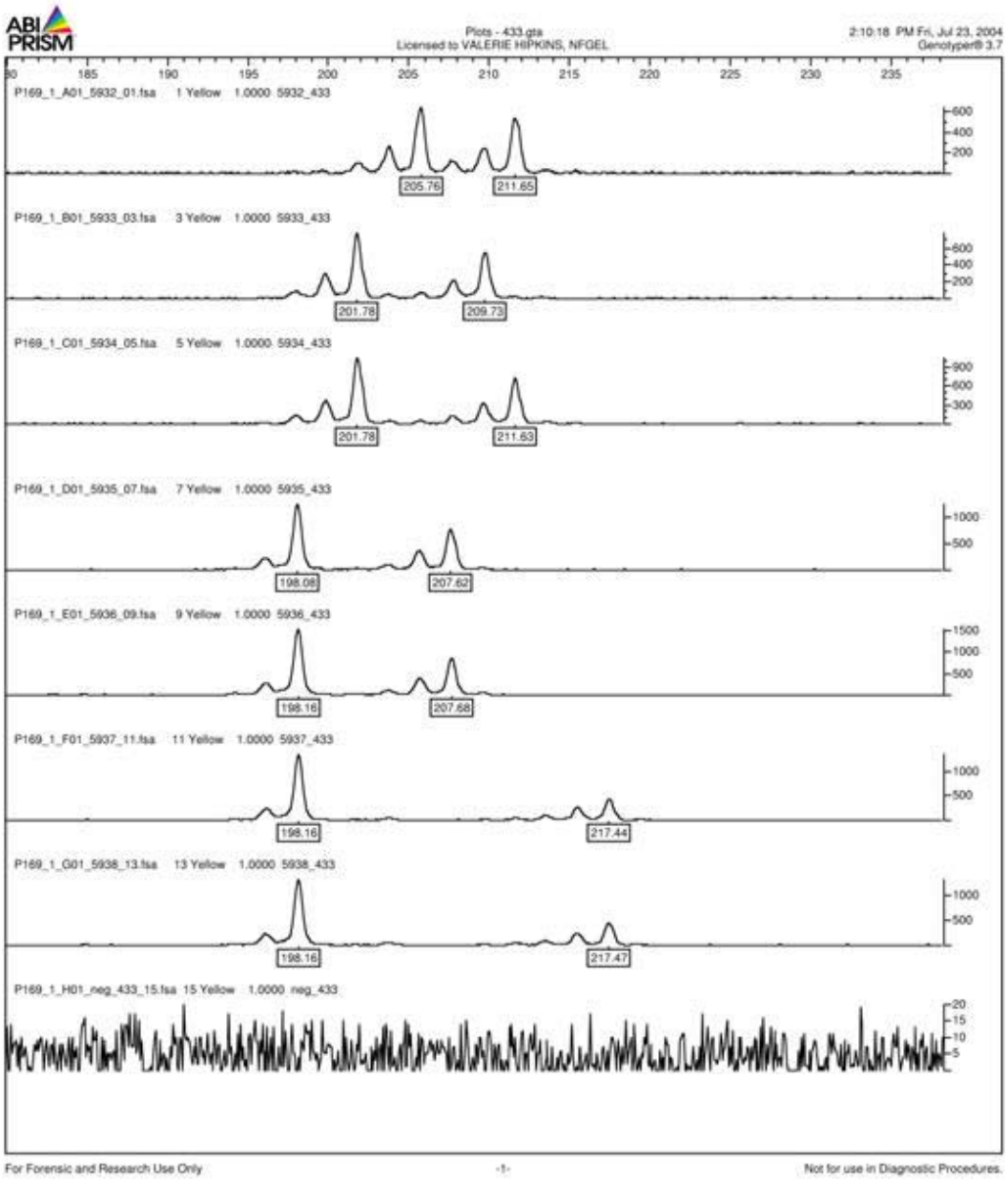

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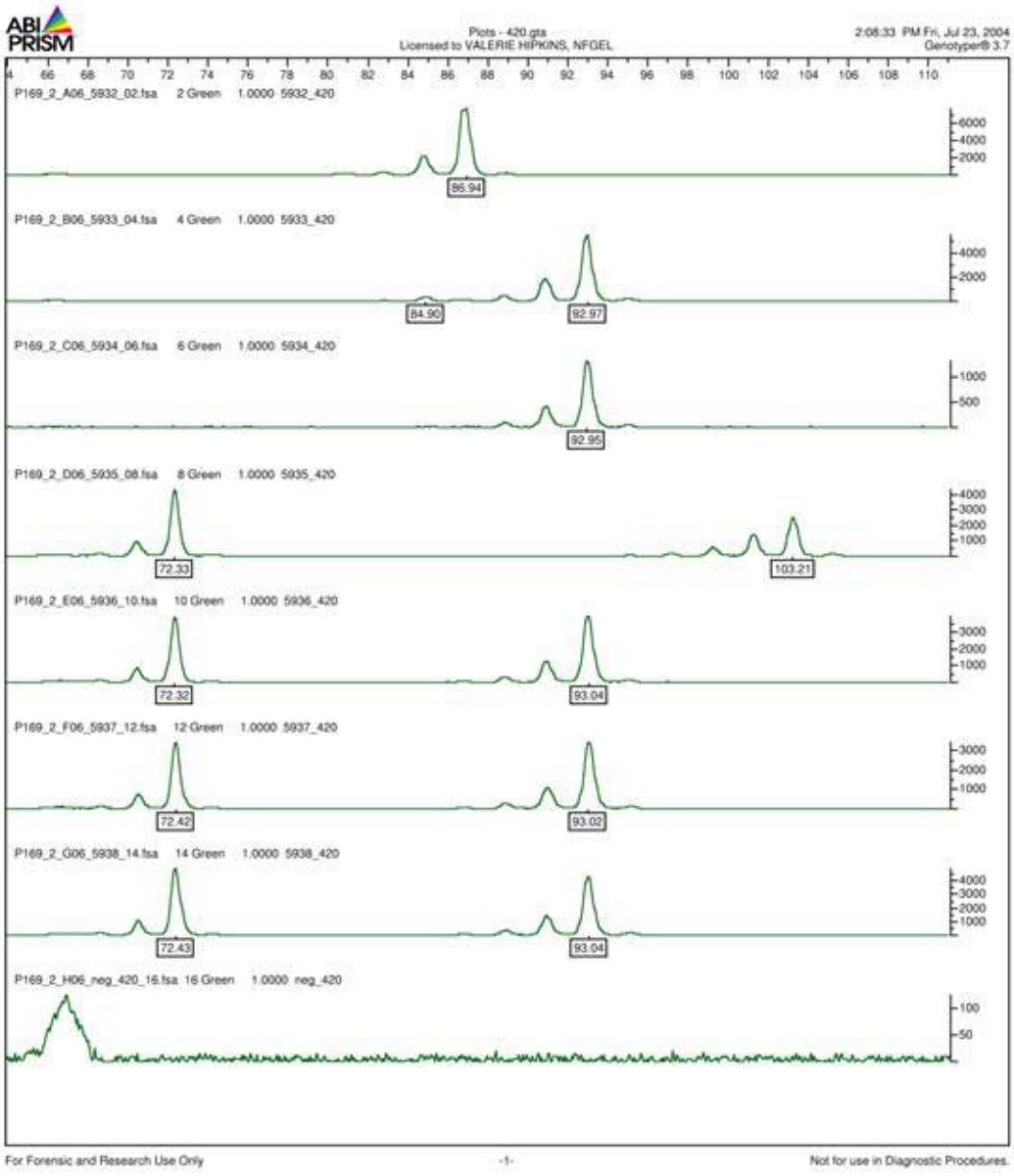

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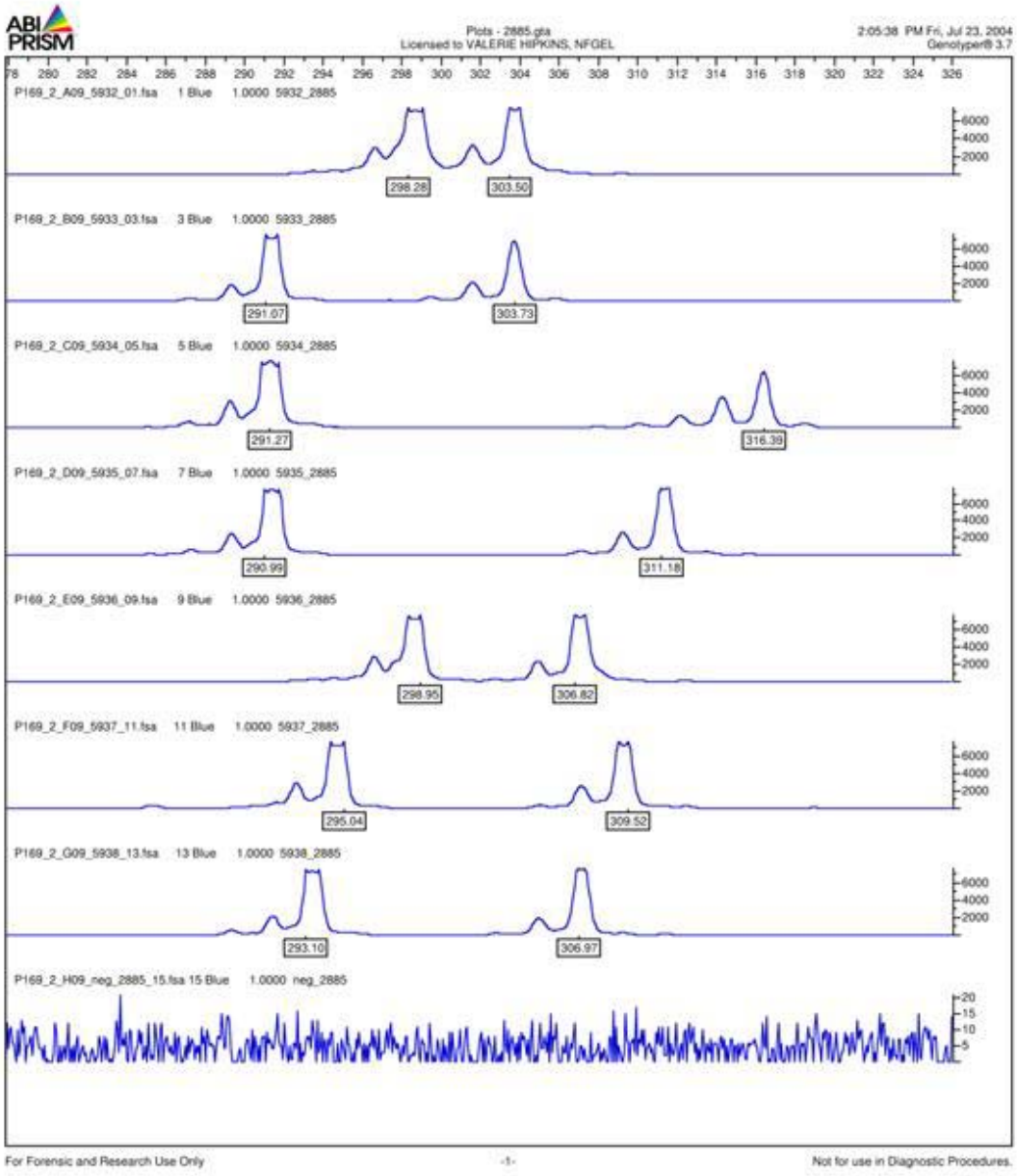

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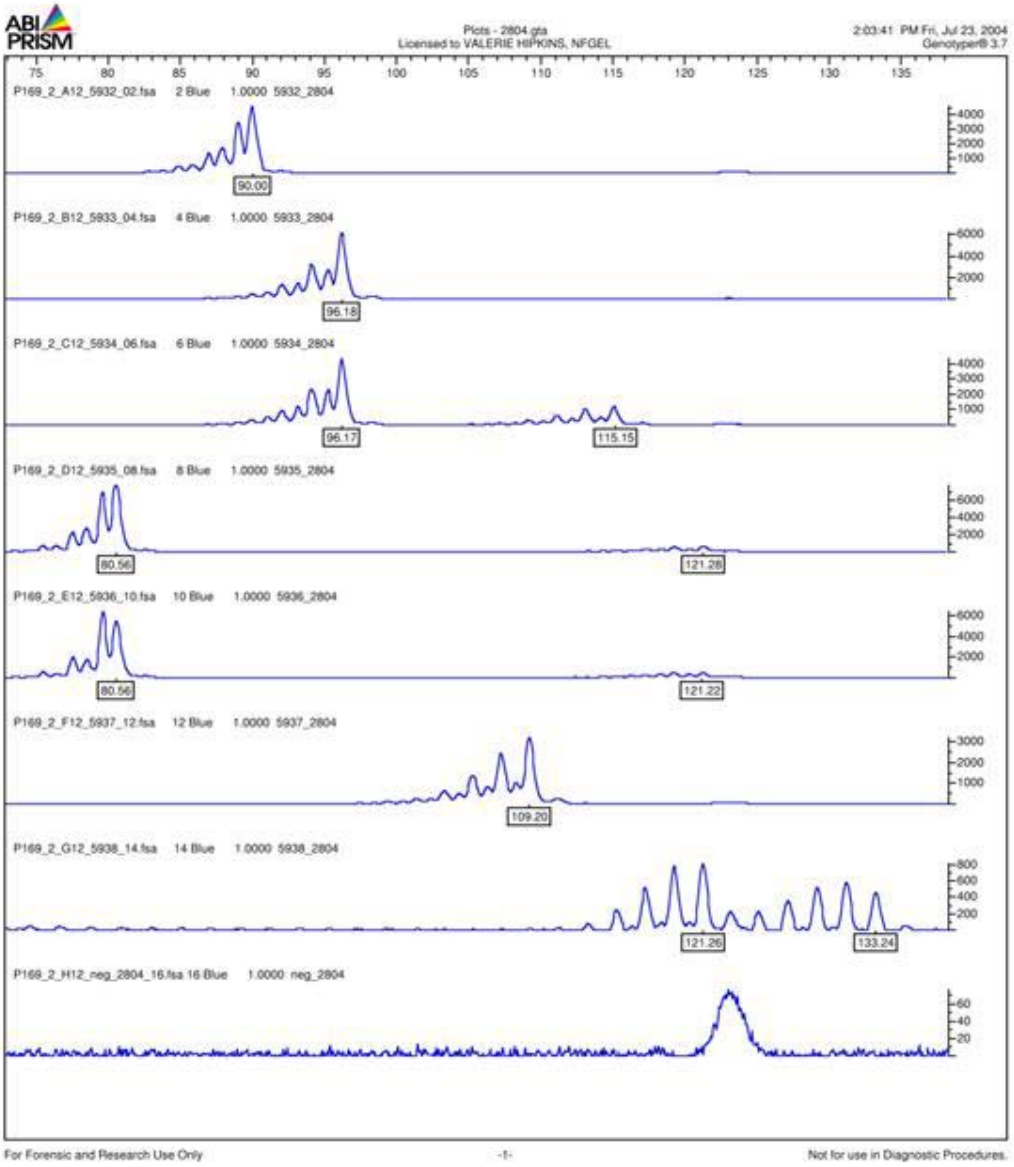

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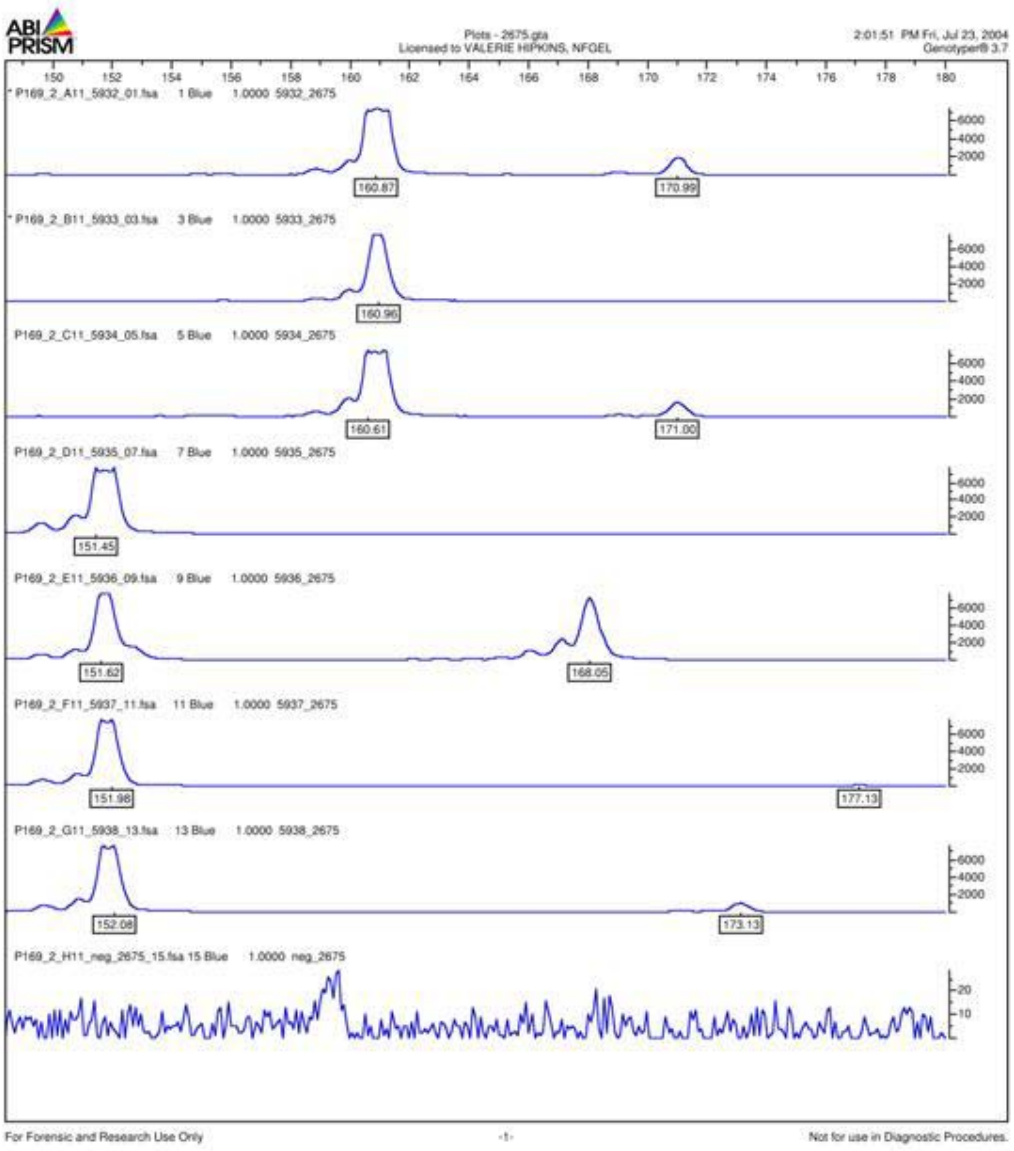

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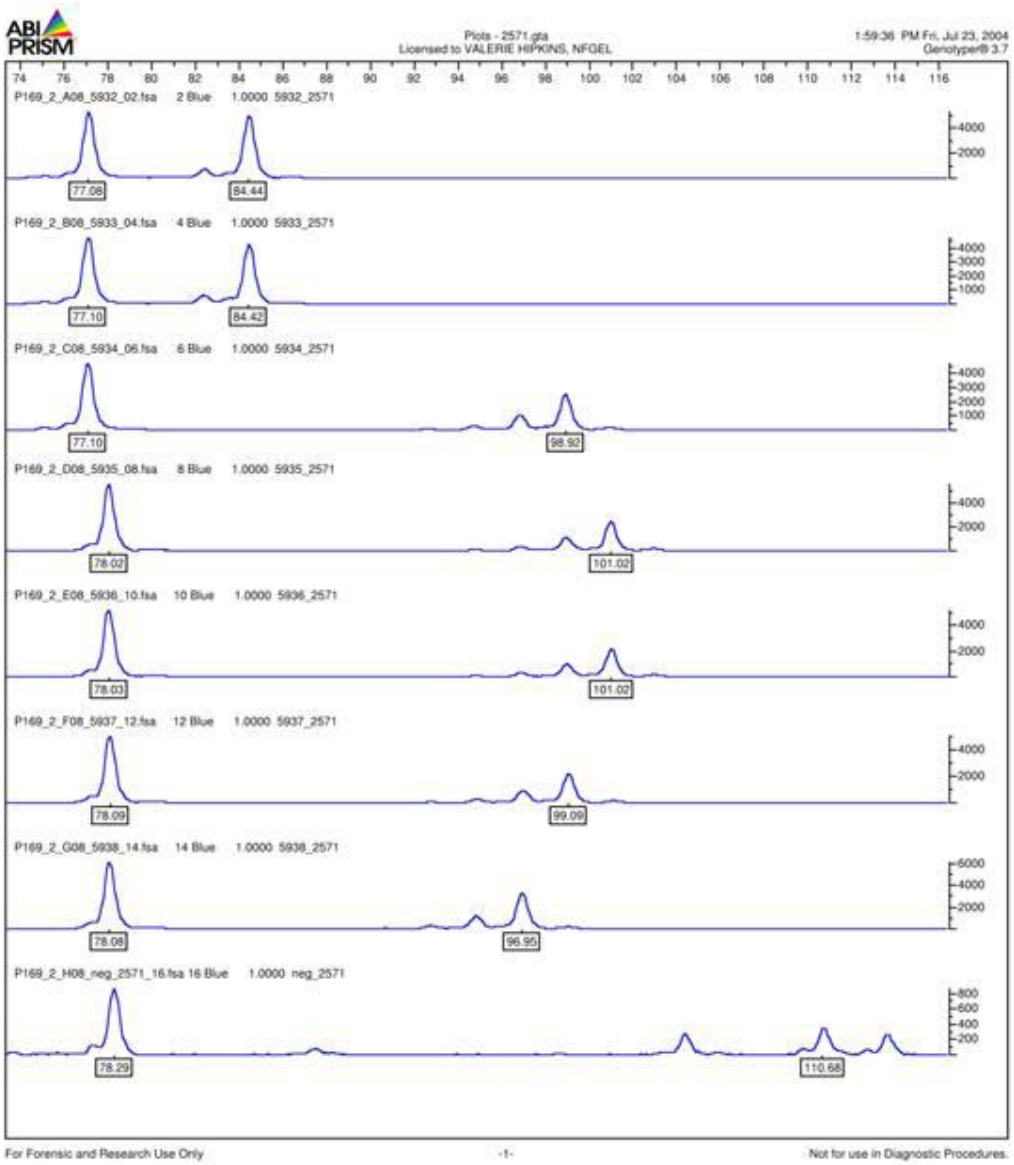

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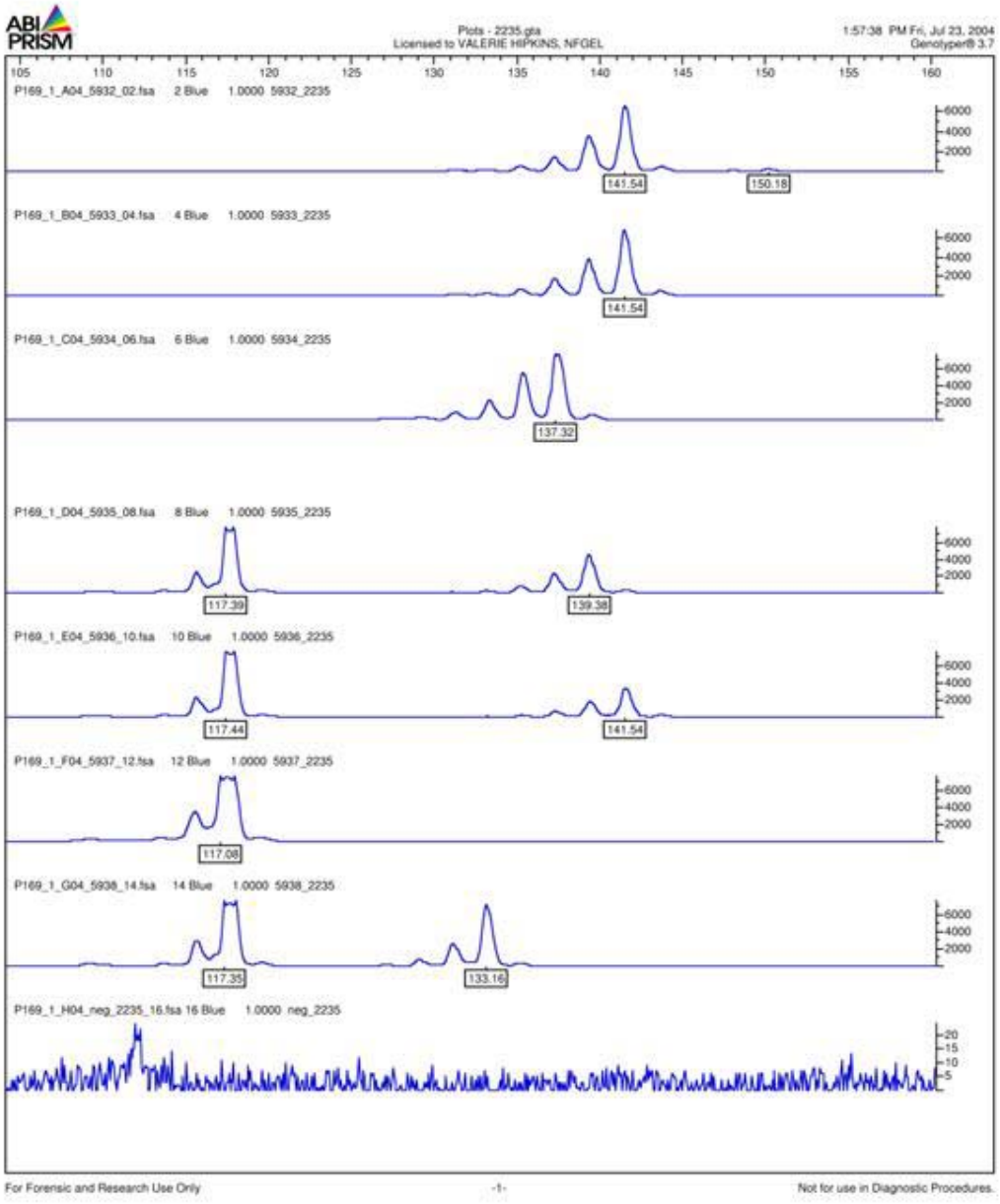

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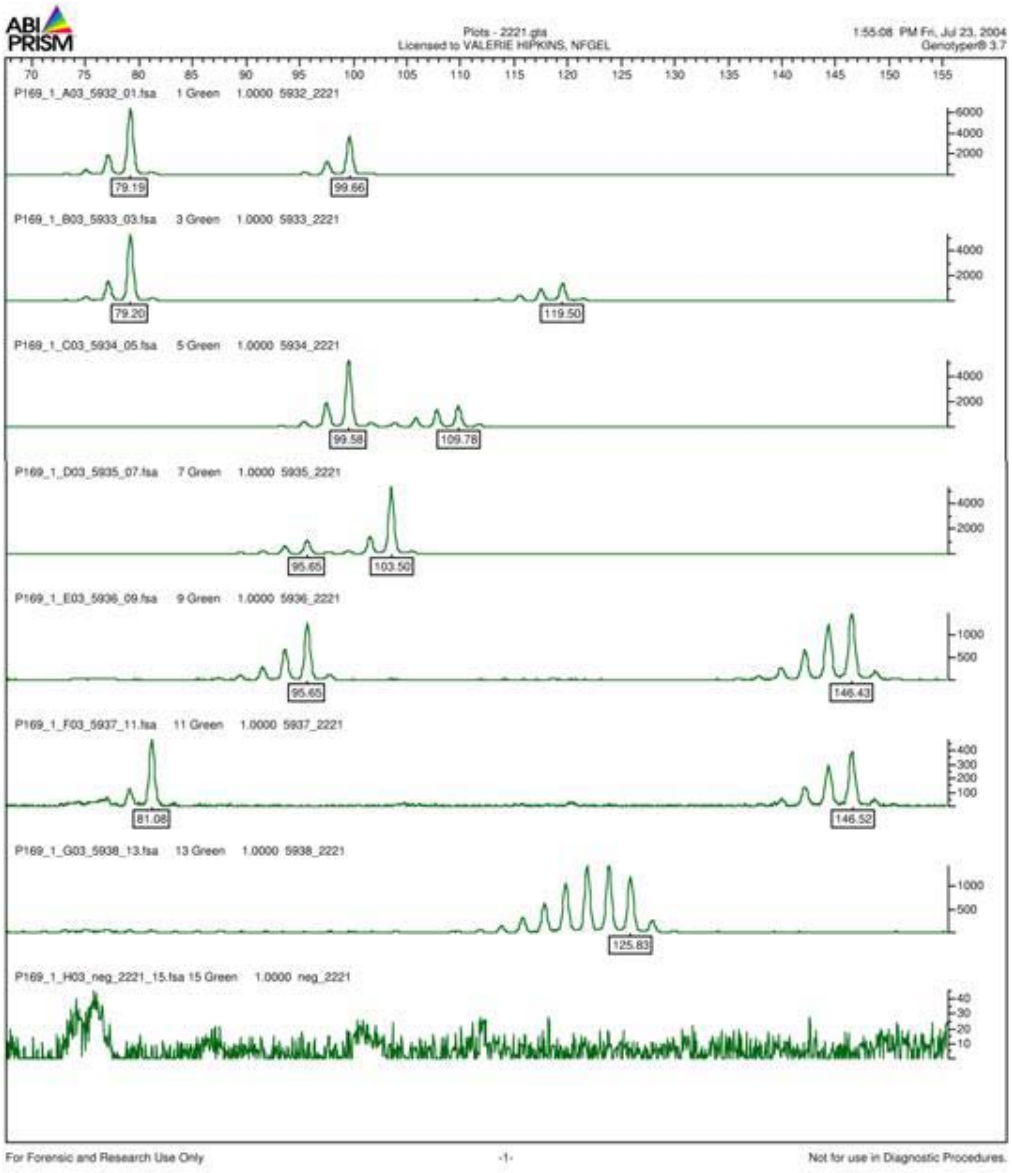

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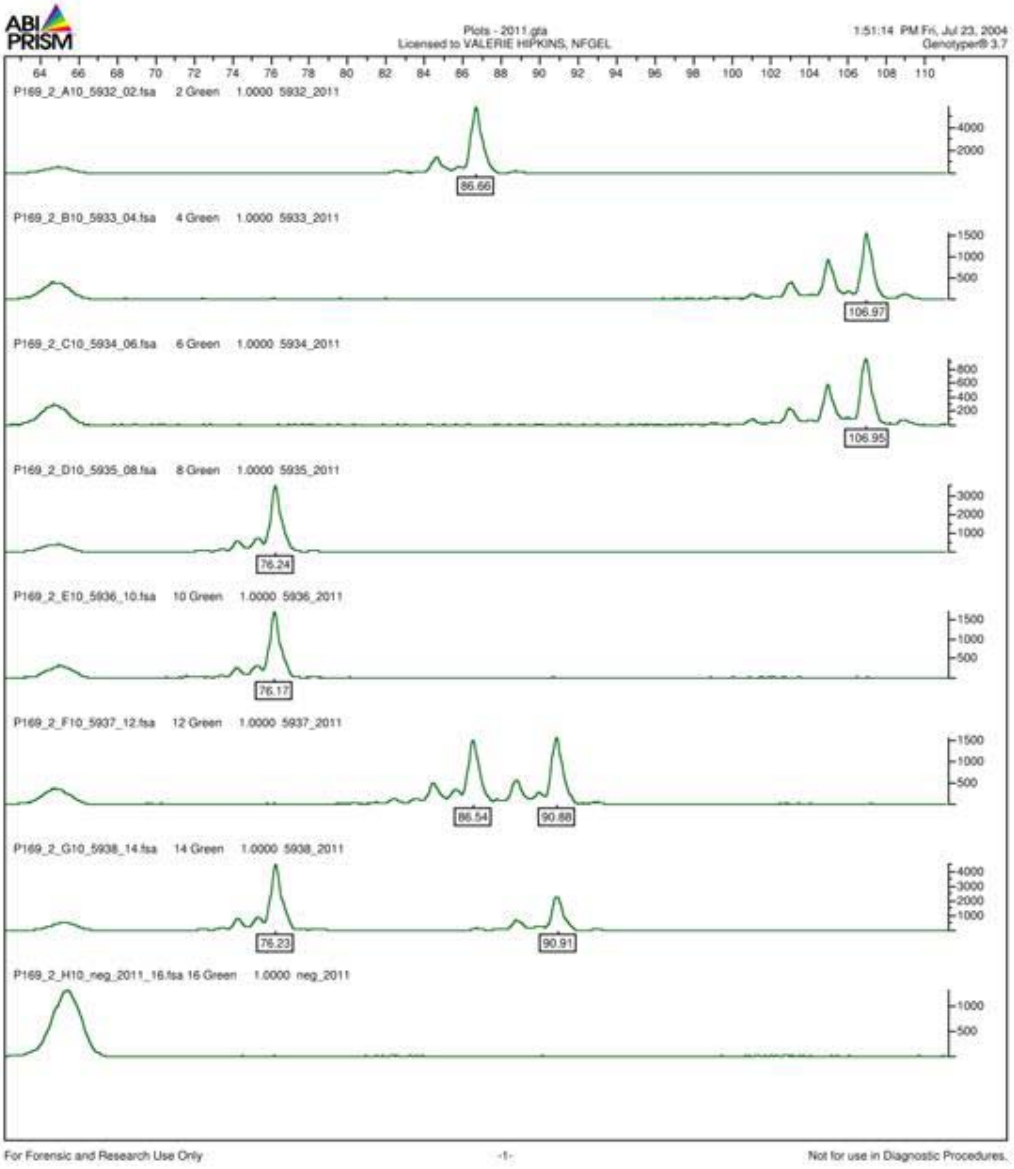

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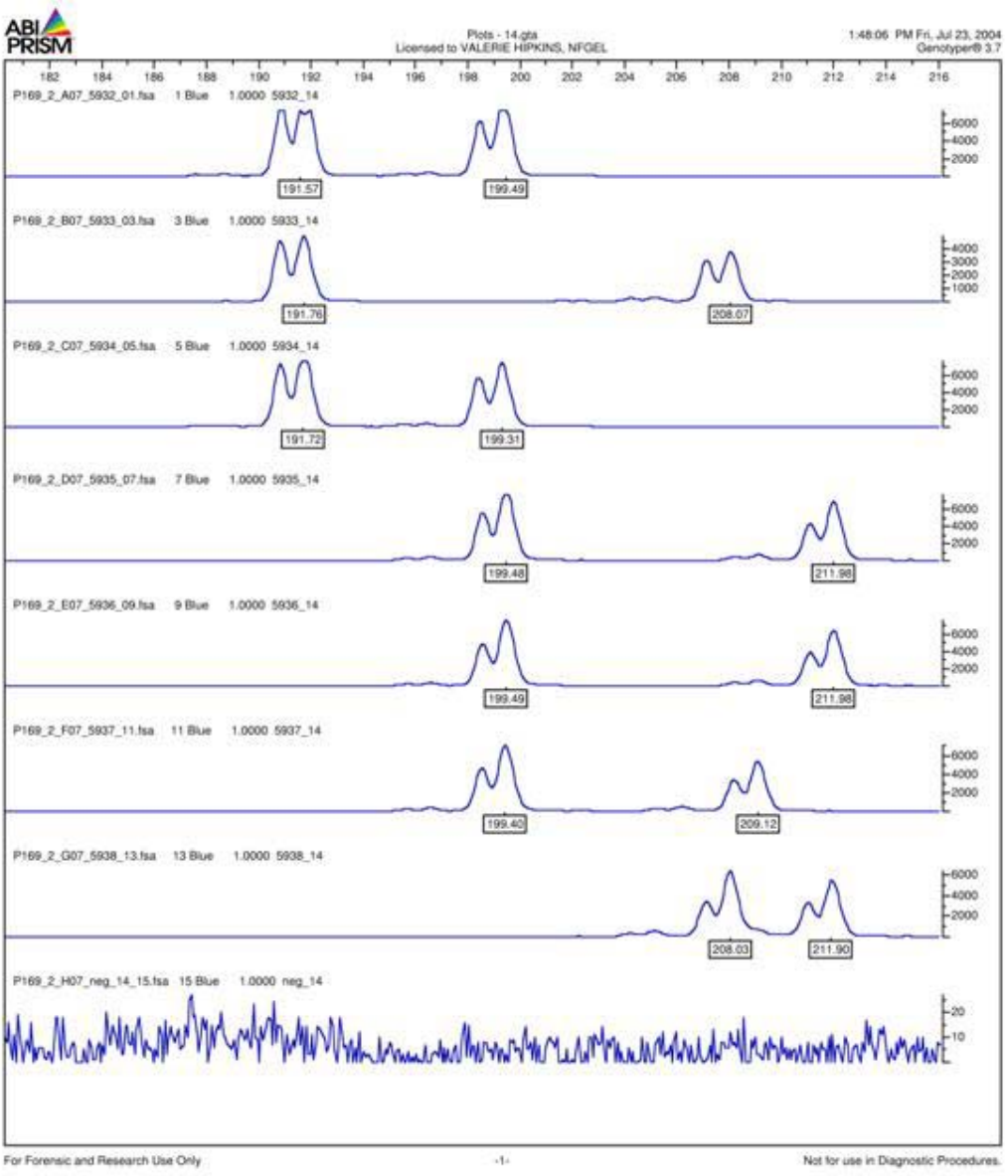

SSR Marker 14.

United States Department of Agriculture **Forest Service** **National Forest Genetic Electrophoresis Laboratory (NFGEL)** **2480 Carson Road Placerville, CA 95667 (530) 622-1609 Voice (530) 622-2633 Fax**

Date: 12/7/04

Angelia Kegley
Dorena Genetic Resource Center
34963 Shoreview Road
Cottage Grove, OR 97424

Dear Angelia:

Here are the results of the isozyme analysis on the Port-Orford-cedar trees you submitted to the lab. The isozymes show low to moderate levels of variation (as they have done in previous POC work here at NFGEL). Therefore, when the data indicate a match in identity, it could be because the samples are really the same individual (or cross), or because the data was not variable enough to detect the difference. I'm hoping Scott and Rich Cronn are successful at coming up with some variable DNA markers in the near future that we can apply to projects such as this.

You'll find the isozyme data attached. Please contact me with any questions! Thanks.

Sincerely,

/s/ *Valerie*

Valerie Hipkins
NFGEL Director

Material Submitted:

NEEDLES

Family	Reason to be tested	Sample ID	NFGEL #
PO-118573 x OP	unexpectedly high mort	Rep 1 sdl 8	8894
		Rep 3 sdl 3	8895
		Rep 3 sdl 9	8896
PO-118573	Parent of PO-118573 x OP	GH2 Box 17	8897
		GH2 Box 18	8898
		GH11 Box 1-6	8899
PO-510015 x PO-DOR-70020	Rr x Rr (higher mort than exp.)[a]	Rep 1 sdl 1	8900
		Rep 3 sdl 6	8901
		Rep 3 sdl 12	8902
PO-510015	Parent of PO-510015 x PO-DOR-70020; also checking to see if all ramets are identical	GH2 Box 23	8903
		GH2 Box 2-2	8904
		GH13 Box 497	8905
PO-DOR-70020	Parent of PO-510015 x PO-DOR-70020; also checking to see if all ramets are identical	GH2 Box 44	8906
		GH2 Box 50	8907
		GH13 Box 476	8908
PO-118569	Checking to see if all ramets are identical	GH2 Box 17 T1	8909
		GH2 Box 17 T2	8910
		GH11 Box 1-6	8911
PO-OSU-CON1	Checking to see if all ramets are identical	GH2 Box 13	8912
		GH13 Box 449	8913
PO-117344	very inconsistent performer; checking to see if all ramets are identical	GH11 Box 1-1	8915
		GH2 Box 7	8914

SEED

Seed ID #	Family	Reason to be tested	NFGEL #
9430567	PO-510044 x PO-117502	rr x Rr (higher mort than exp.)[a]	8884
9430605	PO-OSU-CF1 x PO-117502	Rr x Rr (higher mort than exp.)[a]	8885
9430606	PO-OSU-CF1 x PO-118569	Rr x rr (lower mort than exp.)[a]	8886
9430582	PO-DOR-70080 x PO-117490	unexpectedly high mort	8887
9430194	PO-117490	Seed from parent of 9430582	8888
9430198	PO-117502	Seed from parent of 9430567 and 9430605	8889
118569	PO-118569	Seed from parent of 9430606	8890
9430215	PO-510044	Seed from parent of 9430567	8891
9430235	PO-OSU-CF1	Seed from parent of 9430605 and 9430606	8892
9430583	PO-DOR-70080 x PO-CF1-CON1	Cross from seed parent of 9430582	8893

OBJECTIVE 1: Verify that the ramets of the parents are genetically identical.

(1) PO-118573. All three ramets have matching genotypes.
(2) PO-510015. All three ramets have matching genotypes.
(3) PO-DOR-70020. Ramets "GH2 Box 50" and "GH13 Box 476" have matching genotypes (and match the clonal 70020 genotype determined in Pj. #161). Individual "GH2 Box 44" does not match the genotype of the other two individuals. Individual "GH2 Box 44" is not a ramet of clone PO-DOR-70020.
(4) PO-118569. All three ramets have matching genotypes.
(5) PO-OSU-CON1. Both ramets have matching genotypes.
(6) PO-117344. Both ramets have matching genotypes.

OBJECTIVE 2: Verify that the POC seedlings are the progeny of the parent of record.

(1) PO-118573 x OP. All three tested seedlings can be the progeny of the parent of record. Detected variation in the progeny would have to have been contributed by the OP paternal parents.
(2) PO-510015 x PO-DOR-70020. All three tested seedlings can be the progeny of a cross between the 510015 female parent and the male 70020 genotype represented by ramets "GH2 Box 50" and "GH13 Box 476".
(3) PO-510044 x PO-117502. All ten tested embryos can be the progeny of the parents of record.
(4) PO-OSU-CF1 x PO-117502. All eleven tested embryos can be the progeny of the parents of record.
(5) PO-OSU-CF1 x PO-118569. All eleven tested embryos can be the progeny of the parents of record.
(6) PO-DOR-70080 x PO-117490. The male parent is suspect, and may not actually be 117490. '117490' has a PGI-2 score of '12'. At this locus, we expect to see half the embryos tested (5 out of 10 embryos) contain a '1' allele, the other half contain the '2' allele. All ten embryo's contained a '2' allele from the male parent (the matching meg tissue contained either the Null allele or a '1' allele from the maternal parent). Either '117490' is not the male parent in this cross, or we are just seeing a sampling artifact within the 10 progeny (statistically we expect half the progeny to contain each of the two alleles).
(7) PO-DOR-70080 x PO-CF1-CON1. We are unable to say anything about this cross because the embryos did not resolve. Meg data indicate all progeny came from the same female.

Isozyme data at 19 loci. 12/7/04. (Green highlighted records are data from project #161).

Sample	fest1	lap	pgm1	me7	pgi1	pgi2	ugpp1	tpi1	aat1	aat2	g6pd	gdh	mdh1	mdh2	6pgd1	6pgd2	idh	skd2	fdp1
PO-DOR-70080 x PO-117490 embryo	11	11	11	11	11	2N	11	11	11	11	22	00	11	11	11	11	11	11	11
PO-DOR-70080 x PO-117490 embryo	11	11	11	11	11	2N	11	11	11	11	22	00	11	11	11	11	11	11	11
PO-DOR-70080 x PO-117490 embryo	11	11	11	11	11	12	11	11	11	11	22	00	11	11	11	11	11	11	11
PO-DOR-70080 x PO-117490 embryo	11	11	11	11	11	2N	11	11	11	11	22	00	11	11	11	11	11	11	11
PO-DOR-70080 x PO-117490 embryo	11	11	11	11	11	12	11	11	11	11	22	00	11	11	11	11	11	11	11
PO-DOR-70080 x PO-117490 embryo	11	11	11	11	11	2N	11	11	11	11	22	00	11	11	11	11	11	11	11
PO-DOR-70080 x PO-117490 embryo	11	11	11	11	11	12	11	11	11	11	22	00	11	11	11	11	11	11	11
PO-DOR-70080 x PO-117490 embryo	11	11	11	11	11	2N	11	11	11	11	22	00	11	11	11	11	11	11	11
PO-DOR-70080 x PO-117490 embryo	11	11	11	11	11	2N	11	11	11	11	22	00	11	11	11	11	11	11	11
PO-DOR-70080 x PO-117490 embryo	11	11	11	11	11	2N	11	11	11	11	22	00	11	11	11	11	11	11	11
PO-117490	11	11	11	11	11	12	11	11	11	11	22	13	11	11	11	11	11	11	11
PO-117490 box 12 GH11	11	11	11	11	11	12	11	11	11	11	22	13	11	11	11	11	11	11	11
PO-117490 parent	11	11	11	11	11	12	11	11	11	11	22	13	11	11	11	11	11	11	11
PO-DOR-70080	11	11	11	11	11	1N	11	11	11	11	22	11	10	11	11	11	11	11	11
PO-118569 seed	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-118569 GH2 Box 17 T1	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-118569 GH11 Box 1-6	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-118569 GH11 Box 1-6	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1 x PO-118569 embryo	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1 x PO-118569 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1 x PO-118569 embryo	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1 x PO-118569 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1 x PO-118569 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1 x PO-118569 embryo	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1 x PO-118569 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1 x PO-118569 embryo	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1 x PO-118569 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
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PO-OSU-CF1 x PO-118569 embryo	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-OSU-CF1 box 13 GH2	00	11	00	11	11	11	11	11	11	11	22	11	11	11	00	11	11	11	00

Sample	fest1	lap	pgm1	me7	pgi1	pgi2	ugpp1	tpi1	aat1	aat2	g6pd	gdh	mdh1	mdh2	6pgd1	6pgd2	idh	skd2	fdp1
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	12	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	12	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	12	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	12	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	12	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-OSU-CF1 x PO-117502 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	10	11	11	11	11
PO-510044 x PO-117502 embryo	11	11	11	11	11	11	11	11	11	11	22	11	15	11	11	11	11	11	11
PO-510044 x PO-117502 embryo	11	11	11	11	11	12	11	11	11	11	22	11	15	11	11	11	11	11	11
PO-510044 x PO-117502 embryo	11	11	11	11	11	12	11	11	11	11	22	11	15	11	11	11	11	11	11
PO-510044 x PO-117502 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
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PO-510044 x PO-117502 embryo	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
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PO-117502	11	11	11	11	11	12	11	11	11	11	22	11	11	11	12	11	11	11	11
PO-117502 box 51 GH2	11	11	11	11	11	12	12	11	11	11	22	11	11	11	12	11	11	11	11
PO-117502 box 52 GH2	11	11	11	11	11	12	11	11	11	11	22	11	11	11	12	11	11	11	11
PO-118573 x OP Rep 1 sdl 8	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-118573 x OP Rep 3 sdl 3	11	11	11	11	11	12	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-118573 x OP Rep 3 sdl 9	11	11	11	11	11	11	11	13	11	11	22	11	11	11	11	11	11	11	11
PO-118573 GH2 Box 17	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-118573 GH2 Box 18	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-118573 GH11 Box 1-6	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11

Sample	fest1	lap	pgm1	me7	pgi1	pgi2	ugpp1	tpi1	aat1	aat2	g6pd	gdh	mdh1	mdh2	6pgd1	6pgd2	idh	skd2	fdp1
PO-510015 GH2 Box 23	11	11	11	11	11	11	12	11	11	11	22	11	11	11	11	11	11	11	11
PO-510015 GH2 Box 2-2	11	11	11	11	11	11	12	11	11	11	22	11	11	11	11	11	11	11	11
PO-510015 GH13 Box 497	11	11	11	11	11	11	12	11	11	11	22	11	11	11	11	11	11	11	11
PO-510015 box 22 GH1	11	11	11	11	11	11	12	11	11	11	22	11	11	11	11	11	11	11	11
PO-510015 box 23 GH2	11	11	11	11	11	11	12	11	11	11	22	11	11	11	11	11	11	11	11
PO-DOR-70020 GH2 Box 44	11	11	11	11	11	11	12	11	11	11	22	11	11	11	11	11	11	11	11
PO-DOR-70020 GH2 Box 50	11	11	11	11	11	11	11	11	11	11	22	11	44	11	11	11	11	11	11
PO-DOR-70020 GH13 Box 476	11	11	11	11	11	11	11	11	11	11	22	11	44	11	11	11	11	11	11
PO-DOR-70020 box 476 GH13	00	00	11	00	11	11	11	11	11	11	22	11	44	11	11	11	11	11	11
PO-DOR-70020 box 44 GH2	11	11	11	11	11	11	11	11	11	11	22	11	44	11	11	11	11	11	11
PO-DOR-70020 box 50 GH2	11	11	11	11	11	11	11	11	11	11	22	11	44	11	11	11	11	11	11
PO-510015 x PO-DOR-70020 Rep 1 sdl 1	11	11	11	11	11	11	12	11	11	11	22	11	14	11	11	11	11	11	11
PO-510015 x PO-DOR-70020 Rep 3 sdl 6	11	11	11	11	11	11	12	11	11	11	22	11	14	11	11	11	11	11	11
PO-510015 x PO-DOR-70020 Rep 3 sdl 12	11	11	11	11	11	11	12	11	11	11	22	11	14	11	11	11	11	11	11
PO-OSU-CON1 GH2 Box 13	11	11	11	11	11	12	11	11	11	11	22	11	44	11	11	11	11	11	11
PO-OSU-CON1 GH13 Box 449	11	11	11	11	11	12	11	11	11	11	22	11	44	11	11	11	11	11	11
PO-117344 GH11 Box 1-1	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11
PO-117344 GH2 Box 7	11	11	11	11	11	11	11	11	11	11	22	11	11	11	11	11	11	11	11

United States Department of Agriculture | Forest Service | National Forest Genetic Electrophoresis Laboratory (NFGEL) | 2480 Carson Road Placerville, CA 95667 (530) 622-1609 Voice (530) 622-2633 Fax

NFGEL Report: September 22, 2004

Ploidy Variation in *Acacia koa*

NFGEL Project #186

Contact: Valerie Hipkins, NFGEL Director, vhipkins@fs.fed.us

INTRODUCTION

Currently there is interest in understanding and managing for resistance to koa wilt in *Acacia koa* (koa). Toward this end, there is a need to verify ploidy levels within koa populations.

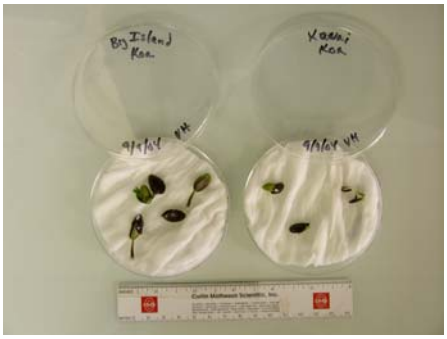
Koa is endemic to Hawaii, and is the only species in its 'group' of *Acacia*'s (the Australian and Pacific Island Group) that is polyploid. There are also known tetraploid *Acacia* species in the 'Asiatic and African Group' and the 'Cosmopolitan Group'. The 'American Group' of *Acacia*'s appears to be comprised solely of diploid species (Darlington and Wylie 1955).

The base chromosome number in *Acacia* is x = 13. Koa is a tetraploid (2n = 4x = 52), while other members of its group are diploid (2n = 2x = 26) (Darlington and Wylie 1955). However, it may be possible that samples used to assess ploidy through early chromosome counts only included part of the range of the species and didn't include the 'subspecies'. Knowing whether all koa populations are tetraploid would help in interpreting disease resistance data and planning any breeding efforts.

METHODS

Twenty-five seed from each of two sources were received at NFGEL on August 2, 2004: "Big Island, Kapa 5" and "Kauai, Kokee l, Lower Ditch" ('**A'**, below).

A. 

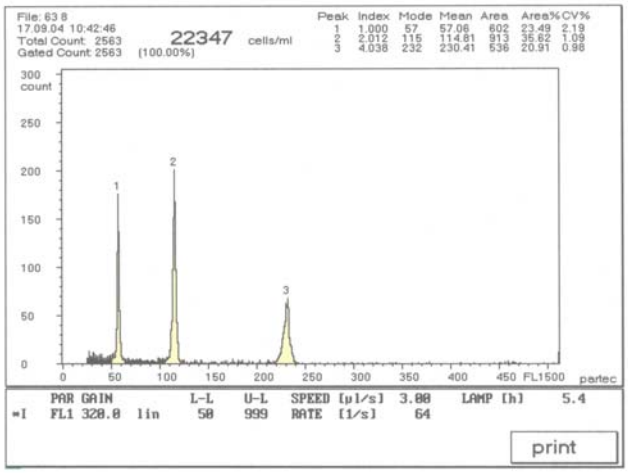
B. 

Six seed per source were scarified (a small cut was made with a scalpel through each seed coat) and soaked in H_2O at room temperature overnight. Three seed per source were prepared for ploidy analysis using the 2-step Partec protocol by extracting approximately ¼ of the seed including endosperm, embryo, and seed membrane. The remaining three seed per source were placed in petri dishes lined with 1%H_2O_2 soaked kimpack and placed in an incubator for germination. All six seeds germinated ('**B**', above), and after 10 days, the root tip from each germinant was prepared for ploidy analysis using the 2-step Partec protocol.

The 2-step Partec protocol consists of the following steps: (1) mince tissue in 0.5ml extraction buffer, (2) incubate at room temperature for five minutes, (3) filter slurry through a green Celltrics filter, (4) incubate at room temperature for fifteen minutes, (5) add 1.5ml stain solution, and (6) read sample on the PA-I using a gain of 320 and LL of 50.

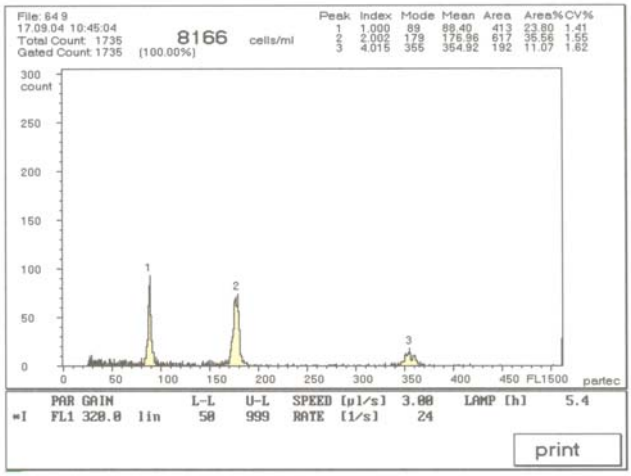
RESULTS

- Ploidy results using seed tissue were the same as those derived from using root tip tissue.
- All six samples analyzed from the Kauai source showed identical ploidy patterns. Without a known diploid or tetraploid control, we cannot definitively identify the ploidy level of these samples. However, we can say they all share the same ploidy level.



Peak position 57 = 2 C
Peak position 115 = 4 C
Peak position 232 = 8 C

- Five of the six samples analyzed from the Big Island source showed the same pattern as that observed in the Kauai source (see figure directly above).
- One of the six samples from the Big Island showed a unique pattern relative to the other 11 samples analyzed (see figure directly below). This sample differs from the other samples in that there appears to be variation in its chromosome number. Therefore, all 12 samples have the same ploidy level, with this one seedling having an apparent chromosome number shift. This sample was also run together with one from Kauai to verify peak positions (plot not shown).



Peak position 89 = 2 C
Peak position 179 = 4 C
Peak position 355 = 8 C

CONCLUSION

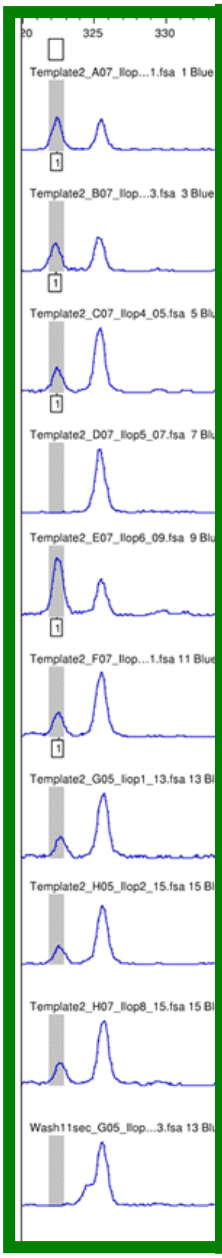
The twelve koa seed analyzed produced the same ploidy pattern indicating that all tested samples have the same ploidy level (likely either diploid or tetraploid). Since the species is thought to be a tetraploid (Darlington and Wylie 1955), it is likely these samples are all tetraploid. If tetraploid, the 2 C peak in the plots represents the tetraploid peak. The 4 C peak is the result of chromosome replication before mitosis. The 8 C peak (and in some samples a hint of a 16 C peak) is observed endopolyploidy. Endopolyploidy is more common in certain tissues, such as root tips. If further ploidy analysis is performed in koa, leaf tissue from seed germinates can be used to minimize the endopolyploid peaks.

One of the twelve seed (one sample from the Big Island source), though sharing the same ploidy level as the other 11 samples analyzed, appears to have a variable chromosome number. This can be the result of many things including chromosome imbalance, aneuploidy, chromosome fragmentation, chromosome fusions, and repeated ploidy events (Briggs and Walters 1997).

REFERENCES

Briggs, D. and S.M. Walters. 1997. Plant Evolution, 3rd Edition. Cambridge University Press.

Darlington, C.D., and A.P. Wylie. 1955. Chromosome Atlas of Flowering Plants. University Press, Aberdeen, Great Britain. 519 pp.



DNA Fingerprinting the "Washington Trees"

Barbara Wilson, Robert Saich, Randy Meyer, Ricardo Hernandez, and Valerie Hipkins

July 19, 2004

Submitted to:

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USDA Forest Service
National Forest Genetics Laboratory (NFGEL)
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Contact: Valerie Hipkins, NFGEL Director
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TABLE OF CONTENTS

SUMMARY

At Mount Vernon, fifteen trees of five species (the "Washington trees") are known to have been planted at the time of George Washington. They include nine American Holly (*Ilex opaca*), one Canadian Hemlock (*Tsuga canadensis*), two Tulip Poplars (*Liriodendron tulipifera*), two White Ash (*Fraxinus americana*), and one White Mulberry (*Morus alba*). These historic trees are the only living witnesses to the life and times of George Washington. However, they are nearing the end of their normal life expectancy. To ensure that these trees will be recognized and protected for future generations, genetic duplicates are being produced through vegetative propagation and grafting.

In order to authenticate the clones of these historically important trees, the National Forest Genetic Electrophoresis Laboratory, USDA Forest Service, investigated the possibility of creating genetic profiles, or genetic (DNA) fingerprints, of each individual Washington tree. DNA is a powerful tool to solve questions of individual identification because of its uniqueness among individuals, immutability (every cell in an individual throughout its life cycle is the same), physical stability (DNA can be recovered and analyzed from tissue), and variability.

This laboratory study evaluated and compared the suitability of isozymes, RAPDs, and AFLPs for establishing genetic fingerprints of "15 Washington Trees". RAPDs and AFLPs both revealed high levels of variation and were successful in distinguishing all individuals tested, with the exception that AFLPs did not distinguish all the Canadian Hemlock samples. Isozyme analysis revealed less variation and was unable to distinguish all individuals.

DNA fingerprints such as those produced here by RAPD and AFLP analysis are well suited for distinguishing genetic individuals of these five tree species. However, at this point in our understanding of genetic variation of trees, the results must be applied cautiously. Certainly, trees with different DNA fingerprints are different. (This is often expressed as "exclusion is absolute.") However, there are two possibilities that would lead to a match between DNA fingerprints generated from different trees. The first is that the fingerprint came from the same tree (or clone). The second is when two different trees coincidentally share the same DNA fingerprint. To address the second possibility, one must statistically estimate how often that DNA fingerprint might occur in the species or population. If enough is known about variation in each species, that question can be answered as, "the chance that a random tree would be found to match our sample due to chance alone is 1 in (say) 20 million." The probability that another tree (not a clone) might have the same DNA fingerprint can be determined only after large-scale sampling of trees in each species. Based on the small number of samples in this study, we were not able to determine the probability of these random matches. To do this, species specific databases would need to be created at the cost of approximately tens to hundreds of thousands of dollars per species.

In keeping with the spirit of scientific innovation exhibited by George Washington, the genetic information revealed in these relatively unstudied species, and the methods used to measure this genetic variation, is the first step toward understanding and protecting an important piece of our nation's genetic heritage.

REPRESENTATIVE
DNA FINGERPRINTS
OF THE
15 'WASHINGTON TREES'

DNA FINGERPRINT

Morus alba
White Mulberry

Fingerprint markers at: RAPD markers OPK1 (blue) and OPK19 (green).

DNA FINGERPRINT

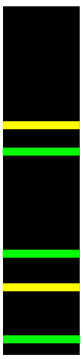
Fraxinus americana
White Ash: WA1

Fingerprint markers at: RAPD markers OPK3 (yellow) and OPK19 (green).

DNA FINGERPRINT

Fraxinus americana
White Ash: WA2

Fingerprint markers at: RAPD markers OPK3 (yellow) and OPK19 (green).



DNA FINGERPRINT

Liriodendron tulipifera
Tulip Poplar: TP1

Fingerprint markers at: RAPD markers OPK2 (red) and OPK19 (green).

DNA FINGERPRINT

Liriodendron tulipifera
Tulip Poplar: TP2

Fingerprint markers at: RAPD markers OPK2 (red) and OPK19 (green).

DNA FINGERPRINT

Tsuga canadensis
Canadian Hemlock

Fingerprint markers at: RAPD markers OPK3 (yellow), OPK13 (purple), and OPK16 (light blue).

DNA FINGERPRINT

Ilex opaca
American Holly: #1

Fingerprint markers at: RAPD markers OPK1 (blue), OPK2 (red), and OPK3 (yellow).

DNA FINGERPRINT

Ilex opaca
American Holly: #2

Fingerprint markers at: RAPD markers OPK1 (blue), OPK2 (red), and OPK3 (yellow).

DNA FINGERPRINT

Ilex opaca
American Holly: #3E

Fingerprint markers at: RAPD markers OPK1 (blue), OPK2 (red), and OPK3 (yellow).

DNA FINGERPRINT

Ilex opaca
American Holly: #3W

Fingerprint markers at: RAPD markers OPK1 (blue), OPK2 (red), and OPK3 (yellow).

DNA FINGERPRINT

Ilex opaca
American Holly: #4

Fingerprint markers at: RAPD markers OPK1 (red), and OPK19 OPK3 (yellow).

DNA FINGERPRINT

Ilex opaca
American Holly: #5

Fingerprint markers at: RAPD markers OPK1 ((blue), OPK2 (red), and OPK3 (yellow).

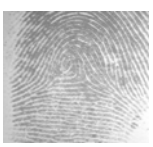
DNA FINGERPRINT

Ilex opaca
American Holly: #6

Fingerprint markers at: RAPD markers OPK1 (blue), OPK2 (red), and OPK3 (yellow).

DNA FINGERPRINT

Ilex opaca
American Holly: #7F



Fingerprint markers at: RAPD markers OPK1 (blue), OPK2 (red), and OPK3 (yellow).

DNA FINGERPRINT

Ilex opaca
American Holly: #7M

Fingerprint markers at: RAPD markers OPK1 (blue), OPK2 (red), and OPK3 (yellow).

METHODS

Three methods of DNA profiling were used: (1) isozyme analysis, (2) RAPD (Random Amplified Polymorphic DNA) analysis, and (3) AFLP (Amplified Fragment Length Polymorphism) analysis. These three methods and some basic genetic terminology are explained in the Appendices of this report. Because the enzymes assayed in isozyme analysis are most active in young tissue, but the DNA extraction methods used at NFGEL are optimized for leaves, two tissue collections were made, one of dormant leaf buds and the other of mature leaves. In addition to the fifteen historic trees from Mount Vernon, other trees of each species were included in the study for comparative purposes, including one White Mulberry and one Tulip Poplar from Thomas Jefferson's estate Monticello (Table 1).

Samples consisting of branches with dormant buds were collected from all species in March 2002. In addition, mature leaves of Tulip Poplar, White Ash, and White Mulberry were collected again in August 2002. Branches from each plant were bagged separately, and samples were shipped on ice to NFGEL. For American Holly and Canadian Hemlock, each sample consisted of six 5 – 8 inch branches with leaves and buds attached. For Tulip Poplar, White Ash, and White Mulberry, the May collections were three 3 – 6 inch dormant branches per individual (no leaves, dormant buds), and the August collections consisted of six 5 - 8 inch branches with leaves attached.

Table 1. Number of trees of each species used in this study. "Washington trees" are the trees that were planted at Mount Vernon under the supervision of George Washington.

Species	Washington Trees		Monticello	Other	Total
	Tree ID	#	#	#	#
American Holly, *Ilex opaca*	1,2,3E,3W,4,5,6,7F,7M	9		1	10
Canadian Hemlock, *Tsuga canadensis*	1	1		4	5
Tulip Poplar, *Liriodendron tulipifera*	TP1,TP2	2	1	5	8
White Ash, *Fraxinus americana*	WA1,WA2	2		4	6
White Mulberry, *Morus alba*	1	1	1		2
Total:		15	2	14	31

RESULTS

Overview

The molecular markers used in this project varied in their ability to identify individuals (Table 2). RAPDs distinguished all the individuals sampled. At least one AFLP primer set distinguished all individuals with the exception of Canadian Hemlock. Isozyme analysis distinguished the fewest individuals. The usefulness of these three methods for identifying individuals was related to the number of loci or DNA fragments the method assessed and the variability found at that site (Table 3). American Holly, Tulip Poplar, and the two White Mulberries had a great deal of variation and individuals were easily recognized by most methods.

Two pairs of American Hollies (3 east and 3 west; 7 female and 7 male) grew as double-trunked trees. DNA fingerprints indicated that each pair consists of two genetically distinct individuals. (This was already apparent in the case of pair 7; American Hollies have separate male and female individuals.) Although AFLPs using *caa* primers did not distinguish trees 3 east and 3 west, RAPDs and AFLPs using the *cac* marker distinguished them easily. The two trunks in each pair may result from the growing together of two old trees originally planted close together, but they are not clones.

Among the Tulip Poplars, tree 3 had its branches somewhat mingled with those of a second tree. Because of concern about which tree had been sampled during the first collection in May 2002, both trees were collected and sent later in the August 2002 shipment. Isozyme analysis indicated that tree 3 had been collected the first time, and the adjacent tree was included in the data set as a different individual, labeled "7".

The final data for each method is included in the next section of this report. Isozyme data is presented in the form of genotype scores, with alleles indicated by letter. RAPD and AFLP data are scored with '1's and '0's. If a band or peak is present, a score of '1' is given; if a band or peak is absent, a score of '0' is assigned. Together, this data forms the DNA "fingerprints".

RAPDs would work well for future DNA fingerprinting of the five species of trees sampled here, and AFLPs would work well for all species except Canadian Hemlock.

Table 2. The number of genetically different individuals of five species recognized by three methods of DNA fingerprinting. (*caa*) and (*cac*) represent two different AFLP primers. * = for one individual, no data could be obtained.

Species	# Samples	RAPDs	AFLP (*caa*)	AFLP (*cac*)	Isozymes
American Holly	10	10	9	10	7
Canadian Hemlock	5	5	2	2	3
Tulip Poplar	8	8	8	7*	7
White Ash	6	6	4*	5*	2
White Mulberry	2	2	2	2	2

Table 3. Genetic variation in five tree species, as revealed by isozymes, RAPDs, and AFLPs. For isozyme analysis, American Holly was treated as a diploid; all heterozygotes, balanced and unbalanced, were treated as heterozygous diploids. For AFLP analysis, results from both primers were combined to give a single genotype. *N* = sample size. *N** = average sample size/locus. Loci*: for isozymes, loci are true genetic loci; for RAPDs and AFLPs, loci are band or peak positions. *P* = percent polymorphic loci (isozymes) or bands (RAPDs and AFLPs). *I* = Shannon-Weaver Information Index (Shannon and Weaver 1949).

Species	Method	N	N*	Loci*	P	I
American Holly	isozymes	10	10.0	16	19%	0.1300
	RAPDs	10	9.1	153	71%	0.3582
	AFLPs	10	10.0	193	33%	0.1659
Canadian Hemlock	isozymes	5	4.8	23	22%	0.1238
	RAPDs	5	4.3	85	20%	0.1151
	AFLPs	5	4.0	51	14%	0.0778
Tulip Poplar	isozymes	8	7.9	25	24.0%	0.1071
	RAPDs	8	7.9	91	31.9%	0.1617
	AFLPs	8	8.0	194	40%	0.2095
White Ash	isozymes	6	5.9	9	11.1%	0.0319
	RAPDs	6	5.8	77	48.0%	0.2315
	AFLPs	6	5.0	176	13%	0.0553
White Mulberry	isozymes	2	1.9	22	36.4%	0.2223
	RAPDs	2	2.0	62	29.0%	0.1756
	AFLPs	2	2.0	26	69%	0.4187

Isozyme Results

Isozyme analysis did not have great enough resolution to distinguish all individuals. This method is inadequate for DNA fingerprinting of the five tree species tested here. However, it reveals variation in all five species for assessing population-level genetic diversity (Table 4). For the three deciduous species tested, dormant buds provided much greater enzyme activity than did mature leaves.

The White Mulberry samples included only two individuals, one from Mount Vernon and one from Monticello, but it was highly variable. The two individuals differed at over a third of sampled loci. It also had the highest variation detected with AFLPs.

For American Holly, the variation is complicated by polyploidy. This species is tetraploid. (It has four sets of chromosomes in each cell, not two like humans and the other sampled trees.) In some measures (e.g. percent polymorphic loci), Holly had low variation. In other ways, it was highly variable. Polyploidy resulted in the observation of unbalanced as well as balanced heterozygotes in the isozymes DIA, TPI-s, and especially LAP. For analysis the Holly genotypes were compressed to diploid status, with both balanced and unbalanced

heterozygotes treated as diploids. Therefore, an excess of heterozygotes was detected (*Ho* >> *He*).

The inbreeding coefficient *F* was zero or negative for four species, and slightly positive for Tulip Poplars, which included a tree (#6) that was a descendent of the Washington tree (TP#1). This means that the trees are more heterozygous than predicted from their allele frequencies; they are more outbred than average. Because each species sample (except American Holly) includes only one or two trees from Washington's time, the value of *F* among the original Washington trees can be calculated meaningfully only for American Holly.

Achieving old age has a large non-genetic component for a tree (depending in part on the environment (eg., weather such as storms; seed germination on a good site) and human activity (eg., decisions to cut down trees)). However, genetics do contribute to survival. In general, old plants and animals average more heterozygosity than young ones. In other words, for each gene, having different alternatives (AB) is usually better than having only one alternative (AA or BB). The American Hollies at Mount Vernon fit this pattern. They are over two centuries old, and they are more heterozygous than predicted from their collective allele frequencies. In fact, at first glance, the inbreeding coefficient *F* (a measure of heterozygosity) is a phenomenal –0.77 (Table 4). However, heterozygosity can easily be overestimated in a tetraploid being treated as diploid. In two loci (DIA and TPI slow), all American Holly individuals are heterozygous, suggesting that what we have defined as the DIA and TPI slow loci each contain two loci. When the alleles of the DIA and TPI are divided into two loci each, and *F* is recalculated, *F* is still –0.38. They are more heterozygous, more outbred, than we calculate average Hollies are likely to be.

Although the Mulberries are outbred, the three old Tulip Poplars (TP#1, TP#2, and Monticello) are not (*F* = 0.09). Comparison of Tulip Poplar #6 with its parent TP#1 confirmed that #6 could be the offspring of TP#1. Tulip Poplar #6 is not the result of self-pollination by TP#1, because it contains two alleles (DIA-C and PGIs-E) that do not occur in TP#1. The isozyme fingerprints of Washington Tulip Poplars indicate with certainty that trees TP#2 and #3 are not parents or offspring of TP#1, because they are homozygous for alleles (AATf-G and SODgdhs-G) not found in TP#1. However, all the other trees (#4 - #7 and Monticello) could be (but might well not be) parents or descendents of TP#1.

Table 4. Genetic variation in five tree species, as revealed by isozymes. American Holly treated as diploid; all heterozygotes, balanced and unbalanced, treated as heterozygous diploids. *N* = sample size. *N** = average sample size/locus. *P* = percent polymorphic loci. *A* = alleles/locus. *Ae* = effective number of alleles/locus (Kimura and Crow 1964). *Ho* = Observed heterozygosity. *He* = expected heterozygosity. *F* = inbreeding coefficient.

Species	N	N*	Loci	P	A	Ae	Ho	He	F
American Holly	10	10.0	16	18.8%	1.19	1.19	0.1750	0.0987	-0.7730
Canadian Hemlock	5	4.8	23	21.7%	1.22	1.15	0.0957	0.0957	0.0000
Tulip Poplar	8	7.9	25	24.0%	1.24	1.10	0.0657	0.0725	0.0938
White Ash	6	5.9	9	11.1%	1.11	1.02	0.0185	0.0185	0.0000
White Mulberry	2	1.9	22	36.4%	1.36	1.27	0.2500	0.2045	-0.2225

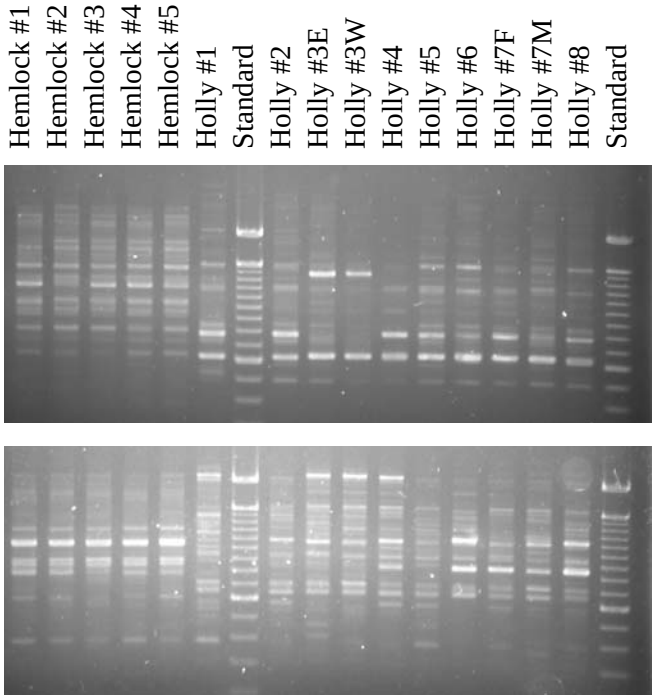
RAPD Results

RAPDs were highly effective markers for DNA fingerprinting the five tree species tested. Most primers tested revealed polymorphic bands that were helpful for DNA fingerprinting (Table 5). All individuals were distinguished. An example of what the RAPD data look like is given in Figure 1. The RAPD DNA patterns look much like a bar-code. Canadian Hemlock exhibited a low level of variability (all the individuals showed a high degree of genetic similarity). American Holly produced many more bands and a higher percentage of polymorphic bands than the other species, probably because American Holly is tetraploid (has twice as many copies of each chromosome as the other species).

Table 5. Summary of results of RAPD analysis of five tree species. American Holly and Canadian Hemlock were screened with 16 primers per species: OPK1 - OPK16. Tulip Poplar, White Ash, and White Mulberry were screen with 12 primers per species: OPK1 - OPK8, OPK17 - OPK20. AMP = number of primers that provided complete amplification. P = number of polymorphic primers. B = number of bands. PB = number of polymorphic bands. R = range of bands per primer. R/P = Range of bands per polymorphic primer.

Species	AMP	P	% P	B	PB	% PB	R	R/P
American Holly	13	13	100%	154	109	71%	3 – 20	3 – 20
Canadian Hemlock	11	7	64%	86	16	19%	4 – 15	4 - 15
Tulip Poplar	11	8	73%	91	29	32%	2 – 17	4 – 17
White Ash	10	6	60%	77	37	48%	2 – 13	5 - 13
White Mulberry	9	6	67%	57	18	32%	3 – 9	6 – 9

Figure 1. An example of RAPD data. Photographs of two RAPD gels (markers) used to generate DNA fingerprints of Canadian Hemlock and American Holly. The 'Standard' is a set of DNA pieces of known size, used for calibration.



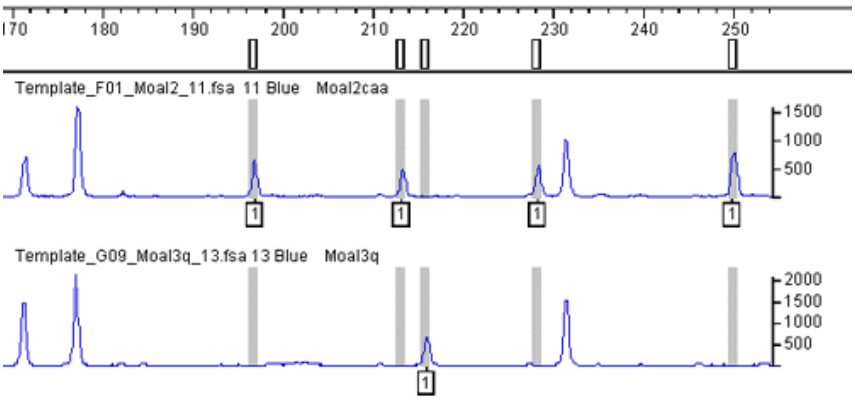
AFLP Results

AFLPs are effective markers for DNA fingerprinting most of the tree species tested. Except in Canadian Hemlock, at least one primer was able to distinguish all the trees in each species. The *caa* AFLP marker detected more variation in the American Holly and White Mulberry than did the *cac* marker. This trend was reversed in the other three species (Table 6). An example of what the AFLP data look like is given in Figure 2.

Table 6. AFLP polymorphism for five tree species. %P = percent of total peaks that are polymorphic.

Species	Total Peaks	Polymorphic peaks	%P
caa primer			
American Holly	62	25	40%
Canadian Hemlock	25	1	4%
Tulip Poplar	95	25	26%
White Ash	62	4	6%
White Mulberry	22	17	77%
cac primer			
American Holly	131	39	30%
Canadian Hemlock	26	6	23%
Tulip Poplar	99	53	54%
White Ash	114	18	16%
White Mulberry	4	1	25%
both primers combined			
American Holly	193	64	33%
Canadian Hemlock	51	7	14%
Tulip Poplar	194	78	40%
White Ash	176	22	13%
White Mulberry	26	18	69%

Figure 2. An example of AFLP data: computer generated traces of one AFLP marker for White Mulberry. Each peak represents a DNA fragment of a certain size. Sizes, in units of base pairs, are given across the top. The upper blue trace is the Mount Vernon tree and the lower trace is the Monticello tree. The trees differ in four peaks, indicated by gray bars.



DNA FINGERPRINT DATA

Isozymes

Isozyme fingerprint data (genotypes) for five tree species. See text of Appendix: Laboratory Techniques for enzyme abbreviations. N = null.

Species	ID	AATf	AATs	ACO	ADH	DIA	FEST	G6PD	GDH	GLYDH	IDH	LAP
American Holly	1	AAAA	NNNN	NNNN	CCCC	EEFG	DDDD	NNNN	NNNN	NNNN	AAAA	AACC
American Holly	2	AAAA	NNNN	NNNN	CCCC	EEFF	DDDD	NNNN	NNNN	NNNN	AAAA	CCCC
American Holly	3E	AAAA	NNNN	NNNN	CCCC	EEFF	DDDD	NNNN	NNNN	NNNN	AAAA	ACCC
American Holly	3W	AAAA	NNNN	NNNN	CCCC	EEFF	DDDD	NNNN	NNNN	NNNN	AAAA	AACC
American Holly	4	AAAA	NNNN	NNNN	CCCC	EEFF	DDDD	NNNN	NNNN	NNNN	AAAA	AACC
American Holly	5	AAAA	NNNN	NNNN	CCCC	EEFF	DDDD	NNNN	NNNN	NNNN	AAAA	AAAA
American Holly	6	AAAA	NNNN	NNNN	CCCC	EEFF	DDDD	NNNN	NNNN	NNNN	AAAA	AAAC
American Holly	7F	AAAA	NNNN	NNNN	CCCC	EEFF	DDDD	NNNN	NNNN	NNNN	AAAA	ACCC
American Holly	7M	AAAA	NNNN	NNNN	CCCC	EEFF	DDDD	NNNN	NNNN	NNNN	AAAA	AAAC
American Holly	8	AAAA	NNNN	NNNN	CCCC	EEFF	DDDD	NNNN	NNNN	NNNN	AAAA	AACC
Canadian Hemlock	1	CC	NN	NN	NN	DF	NN	BB	BB	AA	AA	AB
Canadian Hemlock	2	CD	NN	NN	NN	DF	NN	..	BB	AA	AA	AB
Canadian Hemlock	3	CC	NN	NN	NN	DF	NN	..	BB	AA	AA	AB
Canadian Hemlock	4	CC	NN	NN	NN	DF	NN	BB	BB	AA	AA	AB
Canadian Hemlock	6	CC	NN	NN	NN	DF	NN	..	BB	AA	AA	AB
White Ash	WA1	NN	NN	NN	NN	DD	CC	NN	NN	BB	BC	NN
White Ash	WA2	NN	NN	NN	NN	DD	CC	NN	NN	BB	BB	NN
White Ash	3	NN	NN	NN	NN	DD	CC	NN	NN	BB	BB	NN
White Ash	4	NN	NN	NN	NN	DD	CC	NN	NN	BB	BB	NN
White Ash	5	NN	NN	NN	NN	DD	CC	NN	NN	BB	BB	NN
White Ash	6	NN	NN	NN	NN	DD	CC	NN	NN	..	BB	NN
White Mulberry	Monticello	EE	NN	NN	AA	BB	..	..	AA	NN	EF	BD
White Mulberry	1	EF	NN	NN	AA	BB	AA	..	AA	NN	EF	DD
Tulip Poplar	Monticello	BB	AA	AA	BC	AA	..	AA	CC	NN	DD	NN
Tulip Poplar	TP1	BB	AA	AA	BC	AA	AA	AA	CC	NN	DD	NN
Tulip Poplar	TP2	GG	AA	AA	BC	AA	AA	AA	CC	NN	DD	NN
Tulip Poplar	3	GG	AA	AA	BC	AA	AA	AA	CC	NN	DD	NN
Tulip Poplar	4	BB	AA	AA	BC	AC	AA	AA	CC	NN	DD	NN
Tulip Poplar	5	BB	AA	AA	BC	AA	AB	AA	CC	NN	DD	NN
Tulip Poplar	6	BB	AA	AA	BC	AC	AA	AA	CC	NN	DD	NN
Tulip Poplar	7	BB	AA	..	..	AA	AA	AA	..	NN	..	NN

Isozyme data continued.

Species	ID	MDHd	MDH2	MDH3	MDH4	ME7	6PGDc	6PGDm	6PGDp	PGIf	PGIs
American Holly	1	BBBB	AAAA	GGGG	JJJJ	AAAA	NNNN	NNNN	NNNN	BBBB	NNNN
American Holly	2	BBBB	AAAA	GGGG	JJJJ	AAAA	NNNN	NNNN	NNNN	BBBB	NNNN
American Holly	3E	BBBB	AAAA	GGGG	JJJJ	AAAA	NNNN	NNNN	NNNN	BBBB	NNNN
American Holly	3W	BBBB	AAAA	GGGG	JJJJ	AAAA	NNNN	NNNN	NNNN	BBBB	NNNN
American Holly	4	BBBB	AAAA	GGGG	JJJJ	AAAA	NNNN	NNNN	NNNN	BBBB	NNNN
American Holly	5	BBBB	AAAA	GGGG	JJJJ	AAAA	NNNN	NNNN	NNNN	BBBB	NNNN
American Holly	6	BBBB	AAAA	GGGG	JJJJ	AAAA	NNNN	NNNN	NNNN	BBBB	NNNN
American Holly	7F	BBBB	AAAA	GGGG	JJJJ	AAAA	NNNN	NNNN	NNNN	BBBB	NNNN
American Holly	7M	BBBB	AAAA	GGGG	JJJJ	AAAA	NNNN	NNNN	NNNN	BBBB	NNNN
American Holly	8	BBBB	AAAA	GGGG	JJJJ	AAAA	NNNN	NNNN	NNNN	BBBB	NNNN
Canadian Hemlock	1	DD	CC	GG	II	CC	EE	CC	AA	BB	DD
Canadian Hemlock	2	DD	CC	GG	II	CC	EE	CC	AA	BB	EE
Canadian Hemlock	3	DD	CC	GG	II	CC	EE	CC	AA	BB	EE
Canadian Hemlock	4	DD	CC	GG	II	CC	EE	CC	AA	BB	EE
Canadian Hemlock	6	DD	CC	GG	II	CC	EE	CC	AA	BB	EE
White Ash	WA1	DD	EE	NN	NN	NN	NN	NN	NN	NN	NN
White Ash	WA2	DD	EE	NN	NN	NN	NN	NN	NN	NN	NN
White Ash	3	DD	EE	NN	NN	NN	NN	NN	NN	NN	NN
White Ash	4	DD	EE	NN	NN	NN	NN	NN	NN	NN	NN
White Ash	5	DD	EE	NN	NN	NN	NN	NN	NN	NN	NN
White Ash	6	DD	EE	NN	NN	NN	NN	NN	NN	NN	NN
White Mulberry	Monticello	..	..	NN	NN	BB	BB	DD	GG	AA	FF
White Mulberry	1	GG	GG	NN	NN	BB	BB	DD	GG	AA	DF
Tulip Poplar	Monticello	FF	HH	KK	LL	CC	DD	NN	FF	BB	CC
Tulip Poplar	TP1	FF	HH	KK	LL	CC	DD	NN	FF	BB	CE
Tulip Poplar	TP2	FF	HH	KK	LL	CC	DD	NN	FF	BB	CC
Tulip Poplar	3	FF	HH	KK	LL	CC	DD	NN	FF	BB	CC
Tulip Poplar	4	FF	HH	KK	LL	CC	DD	NN	FF	BB	CC
Tulip Poplar	5	FF	HH	KK	LL	CC	DD	NN	FF	BB	CC
Tulip Poplar	6	FF	HH	KK	LL	CC	DD	NN	FF	BB	CE
Tulip Poplar	7	FF	HH	KK	LL	CC	..	NN	..	BB	CC

Species	ID	PGMf	PGMs	SKD	SODgf	SODgs	TPIf	TPIs	UGPP
American Holly	1	NNNN	NNNN	NNNN	EEEE	IIII	AAAA	EEII	NNNN
American Holly	2	NNNN	NNNN	NNNN	EEEE	IIII	AAAA	EEII	NNNN
American Holly	3E	NNNN	NNNN	NNNN	EEEE	IIII	AAAA	EEII	NNNN
American Holly	3W	NNNN	NNNN	NNNN	EEEE	IIII	AAAA	EEII	NNNN
American Holly	4	NNNN	NNNN	NNNN	EEEE	IIII	AAAA	EEII	NNNN
American Holly	5	NNNN	NNNN	NNNN	EEEE	IIII	AAAA	EEII	NNNN
American Holly	6	NNNN	NNNN	NNNN	EEEE	IIII	AAAA	EEII	NNNN
American Holly	7F	NNNN	NNNN	NNNN	EEEE	IIII	AAAA	EEII	NNNN
American Holly	7M	NNNN	NNNN	NNNN	EEEE	IIII	AAAA	EEII	NNNN
American Holly	8	NNNN	NNNN	NNNN	EEEE	IIII	AAAA	EEEI	NNNN
Canadian Hemlock	1	AA	CC	BB	AA	NN	CC	NN	AA
Canadian Hemlock	2	AA	CC	BB	AA	NN	DD	NN	AA
Canadian Hemlock	3	AA	CC	BB	AA	NN	..	NN	AA
Canadian Hemlock	4	AA	CC	BB	AA	NN	..	NN	AA
Canadian Hemlock	6	AA	CC	BB	AA	NN	DD	NN	AA
White Ash	WA1	NN	NN	NN	NN	JJ	GG	HH	NN
White Ash	WA2	NN	NN	NN	NN	JJ	GG	HH	NN
White Ash	3	NN	NN	NN	NN	JJ	GG	HH	NN
White Ash	4	NN	NN	NN	NN	JJ	GG	HH	NN
White Ash	5	NN	NN	NN	NN	JJ	GG	HH	NN
White Ash	6	NN	NN	NN	NN	JJ	GG	HH	NN
White Mulberry	Monticello	BB	NN	CD	AB	FF	BD	FF	BC
White Mulberry	1	BB	NN	CD	BB	FF	BD	FF	BB
Tulip Poplar	Monticello	BB	NN	AA	CC	HH	JJ	KK	AA
Tulip Poplar	TP1	BB	NN	AA	CC	HH	JJ	KK	AA
Tulip Poplar	TP2	BB	NN	AA	CC	HH	JJ	KK	AA
Tulip Poplar	3	BB	NN	AA	CC	GG	JJ	KK	AA
Tulip Poplar	4	BB	NN	AA	CC	HH	JJ	KK	AA
Tulip Poplar	5	BB	NN	AA	CC	HH	JJ	KK	AA
Tulip Poplar	6	BB	NN	AA	CC	HH	JJ	KK	AA
Tulip Poplar	7	..	NN	AA	..	..	JJ	KK	AA

RAPDs

RAPD fingerprint data presented by species. Unit for size is base pair. 1 = band present. 0 = band absent. ---- = no data.

AMERICAN HOLLY (OPK RAPD primers)

Primer:	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3	3
Size:	1510	1400	1300	1100	750	695	515	430	400	1460	1425	800	750	695	575	475	380	325	1510	1465	1410
Indiv.																					
1	1	1	0	1	1	1	1	1	0	1	0	0	1	0	1	1	0	0	1	1	0
2	0	1	0	1	1	1	1	0	1	1	1	1	0	0	1	1	0	1	0	0	1
3E	0	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1
3W	0	0	1	0	0	0	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1
4	0	0	0	1	0	1	1	0	1	1	1	0	1	1	0	1	0	1	1	1	1
5	0	1	0	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1	0	0	1
6	0	1	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	0	1	0
7F	0	0	0	1	1	1	1	0	1	1	1	0	1	0	1	1	0	1	0	0	1
7M	0	0	0	1	1	1	1	0	1	1	1	0	1	1	1	1	0	1	0	0	1
8	0	1	0	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1	0	0	1

Primer:	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	4	4	4	4
Size:	1390	1300	1250	1200	1075	1025	1000	950	900	850	750	600	540	475	375	320	285	1540	1510	1465	1450
Indiv.																					
1	0	1	0	0	1	0	1	0	1	1	0	1	1	1	0	0	1	1	1	0	1
2	0	1	1	0	0	0	1	0	0	1	0	1	1	1	0	0	0	1	1	0	1
3E	0	1	0	1	0	0	1	0	0	1	0	1	1	0	1	1	0	1	1	0	1
3W	0	1	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	1	1	0	1
4	1	1	0	0	0	0	1	1	0	1	1	1	1	1	0	0	1	0	1	1	1
5	0	0	0	0	0	0	1	0	0	1	0	1	1	1	0	0	0	0	1	1	1
6	1	1	0	0	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	1
7F	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	1
7M	0	1	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	1	0	1
8	0	0	0	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	1

Primer:	4	4	4	4	4	4	4	4	4	4	8	8	8	8	8	8	8	8	8	8
Size:	1435	1400	1100	1030	930	790	725	650	575	425	1510	1480	1420	1300	1250	1150	925	800	750	705
Indiv.																				
1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	0
2	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1
3E	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1
3W	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1
4	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1
6	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0	0	0
7F	1	0	1	1	1	1	1	0	1	1	0	1	1	1	1	0	1	1	0	0
7M	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	1
8	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0

Primer:	8	8	8	8	8	9	9	9	9	9	9	9	9	9	9	9	9	10	10	10	10
Size:	575	500	425	360	290	1580	1570	1535	1520	1500	1490	1480	1300	1000	825	775	415	1525	1515	1455	1435
Indiv.																					
1	0	1	0	1	1	1	0	0	1	1	0	0	1	1	1	0	1	1	1	1	1
2	0	0	0	1	1	1	0	1	0	1	0	0	1	1	1	0	1	1	1	1	1
3E	1	1	0	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1
3W	1	1	0	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
4	1	0	1	0	0	1	1	1	0	1	1	0	1	1	1	0	1	1	1	1	0
5	1	1	0	1	0	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
6	1	0	0	1	0	1	0	1	0	1	1	0	1	1	1	1	1	1	1	1	0
7F	1	0	0	0	0	0	1	1	0	1	1	1	1	1	1	0	1	0	1	1	0
7M	1	0	1	1	0	0	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1
8	0	1	0	1	0	0	1	0	0	1	1	0	1	1	1	0	1	0	1	1	1

AMERICAN HOLLY CON'T (RAPDS)

Primer:	10	10	10	10	11	11	11	11	11	11	11	11	11	11	11	12	12
Size:	1410	1300	1150	485	1510	1500	1440	1320	1270	1200	1025	935	700	280	180	1435	1350
Indiv.:																	
1	0	0	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1
2	0	1	1	1	1	1	0	0	1	1	1	1	1	0	0	1	1
3E	1	0	1	0	1	1	1	1	1	1	1	1	0	0	0	1	1
3W	1	0	1	0	0	1	1	1	1	1	1	1	0	0	0	1	1
4	0	1	1	1	1	1	1	0	1	0	1	1	1	0	0	1	1
5	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1
6	0	0	1	1	1	1	1	0	1	0	1	1	1	0	0	1	1
7F	0	1	1	1	1	1	1	0	1	0	1	1	0	0	0	1	1
7M	0	0	1	1	1	1	1	0	1	0	1	1	1	0	0	1	1
8	0	0	1	0	0	1	0	1	1	0	1	1	1	0	0	1	1

Primer:	12	12	12	12	12	12	12	13	13	13	13	13	13	13	13	13	14
Size:	1080	1020	825	745	675	600	580	1600	1560	1530	1495	1440	1400	875	450	350	250	1580
Indiv.																		
1	1	0	1	1	1	1	1	---	---	---	---	---	---	---	---	---	---	1
2	1	0	1	1	1	1	1	---	---	---	---	---	---	---	---	---	---	1
3E	1	0	1	1	0	1	1	---	---	---	---	---	---	---	---	---	---	0
3W	1	0	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0
4	1	1	1	1	1	1	1	---	---	---	---	---	---	---	---	---	---	0
5	1	1	1	1	1	1	1	---	---	---	---	---	---	---	---	---	---	0
6	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	0
7F	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0
7M	1	1	1	1	1	1	1	---	---	---	---	---	---	---	---	---	---	0
8	1	0	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	---

Primer:	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
Size:	1550	1500	1455	1440	1405	1395	1350	1300	1160	1110	1090	1060	1030	950	890	800	1580	605
Indiv.																		
1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	1	1
2	1	0	1	1	0	0	1	0	0	0	0	1	0	1	1	0	1	0
3E	1	1	1	0	1	0	1	1	0	1	0	0	0	1	0	0	0	1
3W	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1
5	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	1
6	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0
7F	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1
7M	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Primer:	14	15	15	15	15	15	15	15	15	15	15	15	15	15	15	16	16	16
Size:	550	1500	1475	1450	1425	1260	1130	1050	920	825	775	560	550	380	370	500	390	340
Indiv.																		
1	1	1	1	1	0	1	0	1	0	0	1	1	0	0	0	0	0	1
2	1	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	0	1
3E	1	1	1	1	0	0	1	1	1	0	0	0	1	0	0	0	0	1
3W	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	0	0	1
4	1	1	1	1	0	1	1	1	1	1	0	1	0	0	1	0	0	1
5	1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	0	1
6	0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	0	1
7F	1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	0	1
7M	0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	0	1
8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1	1	1

CANADIAN HEMLOCK (OPK RAPD Primers).

Primer:	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2
Size:	1475	1450	1375	1200	1125	950	875	825	700	625	525	1475	1450	1250	1150	950	825	640
Indiv.																		
1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
2	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1

Primer:	2	3	3	3	3	3	3	4	4	4	4	4	4	9	9	9	9	9
Size:	575	1100	950	800	700	500	290	1025	875	850	790	550	475	1530	1460	1005	890	430
Indiv.																		
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Primer:	9	10	10	10	10	10	10	10	11	11	11	11	13	13	13	13	13
Size:	300	1475	1225	1065	900	800	600	450	915	885	725	525	1475	1450	1300	1200	1050
Indiv.																	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
3	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Primer:	13	13	13	13	13	14	14	14	14	14	14	14	14	14	14	14
Size:	940	910	650	350	240	1535	1510	1475	1425	1210	1075	950	825	750	710	650	600
Indiv.																	
1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0
2	1	1	1	1	1	---	---	---	---	---	---	---	---	---	---	---	---
3	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1
4	1	1	1	1	0	---	---	---	---	---	---	---	---	---	---	---	---
5	1	1	1	1	1	---	---	---	---	---	---	---	---	---	---	---	---

Primer:	14	14	14	15	15	15	15	15	15	15	15	16	16	16	16
Size:	530	460	430	1460	1410	1220	1000	840	670	510	440	660	598	440	405
Indiv.															
1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
2	---	---	---	---	---	---	---	---	---	---	---	1	1	1	0
3	1	1	1	---	---	---	---	---	---	---	---	1	1	1	1
4	---	---	---	1	1	1	1	1	1	0	0	1	1	1	0
5	---	---	---	1	1	1	1	1	0	0	0	1	1	1	0

TULIP POPLAR (OPK RAPD primers).

Primer:	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2
Size:	1630	1572	1472	1364	1321	1242	1198	1162	1047	853	781	695	529	1920	1860	1790	1650
Indiv.																	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
2	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
4	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1
6	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
Monticello	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1

Primer:	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3
Size:	1480	1430	1350	1220	1120	1080	1040	810	780	680	580	480	415	1700	1660	1460	1420	1350
Indiv.																		
1	1	1	1	0	0	1	0	1	1	1	1	0	1	1	1	1	1	0
2	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	1	0
3	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	1
4	1	1	1	0	1	0	0	1	0	0	0	0	1	1	1	0	1	0
5	1	0	1	0	1	0	1	1	0	1	0	1	0	1	1	0	1	0
6	1	1	1	0	0	1	0	1	0	0	0	0	1	1	1	0	1	0
7	1	1	1	0	1	0	0	1	0	0	0	0	0	1	1	1	1	0
Monticello	1	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	0

Primer:	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4
Size:	1180	1110	890	810	710	490	405	380	265	1640	1610	1470	1050	790	740	680	510	460
Indiv.																		
1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0
4	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
5	1	1	1	0	1	1	1	0	1	0	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0
7	1	1	1	0	1	1	1	0	1	0	1	1	1	1	1	1	1	1
Monticello	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1	1

Primer:	6	6	6	6	6	6	6	7	7	7	7	7	7	8	8	8	8	8	8
Size:	1425	1270	1170	780	665	600	465	1575	1420	1100	965	705	565	1480	1460	1400	1200	1140	670
Indiv.																			
1	1	1	1	1	1	1	1	---	--	---	---	---	---	0	1	1	0	1	1
2	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
6	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Monticello	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Primer:	17	17	17	17	17	17	18	18	18	18	19	19	19	19	19	19	19	20	20
Size:	1575	1405	1110	875	640	485	1410	1380	1020	690	1710	1495	1410	1030	840	650	290	1410	920
Indiv.																			
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
2	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
4	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1
7	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1
Monticello	1	0	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1

WHITE ASH (OPK RAPD primers).

Primer:	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
Size:	1950	1800	1520	1320	1010	830	735	580	1460	1350	1280	1135	925	770	650	580	475	325
Indiv.																		
1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1
2	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1
3	1	1	1	1	1	1	1	1	---	---	---	---	---	---	---	---	---	---
4	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0
5	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
6	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1

Primer:	3	3	3	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4
Size:	1980	1800	1680	1520	1480	1325	1277	1200	1150	1010	900	825	720	1525	1430	1350	1105	960
Indiv.																		
1	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	1	1	1
2	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	0	1	1	1	1	0	---	---	---	---	---
4	1	1	0	1	0	1	1	1	0	0	1	1	0	1	1	1	1	1
5	1	1	0	1	0	1	0	0	0	0	1	1	0	1	1	1	1	1
6	1	1	0	1	0	1	1	1	0	0	0	1	0	1	1	1	1	1

Primer:	4	4	6	6	7	7	8	8	8	8	8	17	17	17	17	17	17
Size:	845	810	1170	850	900	380	1505	1240	1040	900	480	1600	1390	1200	1125	850	655
Indiv.																	
1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
3	---	---	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0
4	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0
6	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0

Primer:	17	19	19	19	19	19	19	19	19	19	19	19	19	20	20	20	20
Size:	480	1570	1440	1360	1220	880	860	820	800	750	665	475	350	1520	1425	1335	1245
Indiv.																	
1	0	1	1	1	0	0	0	0	0	1	0	1	0	1	1	1	0
2	0	1	1	1	1	1	0	0	1	0	0	1	1	1	0	0	1
3	0	1	1	1	0	0	0	1	0	1	0	1	0	1	0	0	0
4	1	1	1	1	0	0	0	0	0	1	0	1	1	1	0	0	1
5	0	1	1	1	0	0	1	0	0	1	0	1	0	1	0	0	1
6	0	1	1	1	1	1	0	0	0	1	1	1	1	1	0	0	1

Primer:	20	20	20	20	20	20	20
Size:	1100	970	905	830	767	690	490
Indiv.							
1	0	1	1	1	0	1	0
2	1	1	1	1	1	1	0
3	0	1	1	1	0	1	0
4	0	1	1	1	0	0	1
5	0	1	1	1	0	0	0
6	0	1	0	1	0	1	0

WHITE MULBERRY (OPK RAPD primers).

Primer:	1	1	1	1	1	1	1	2	2	2	2	2	4	4	4	4	4	4
Size:	1800	1200	790	650	525	340	335	1693	1480	1250	685	540	1395	1275	916	790	610	550
Individual:																		
Mt. Vernon	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
Monticello	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	0

Primer:	4	4	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7
Size:	505	450	1521	1370	1246	1155	1085	810	780	470	396	1664	1570	1420	1405	1260	915	450
Individual:																		
Mt. Vernon	1	1	1	1	1	1	1	0	1	0	1	1	1	1	0	1	1	1
Monticello	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	0	1	1

Primer:	8	8	8	17	17	17	17	17	17	17	17	17	19	19	19	19
Size:	790	275	113	1505	1460	1400	1230	1036	880	750	640	535	1445	1325	1268	1185
Individual:																
Mt. Vernon	1	1	1	1	1	1	0	0	0	1	0	1	1	1	1	1
Monticello	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1

Primer:	19	19	20	20	20
Size:	580	485	1080	625	270
Individual:					
Mt. Vernon	1	1	1	1	1
Monticello	1	1	1	1	1

AFLPs

AFLP fingerprint data presented by species. Unit for size is base pair. 1 = band present. 0 = band absent. -- = no data.

AMERICAN HOLLY

(AFLP *caa* primer: 37 monomorphic peaks not shown).

size	104	105	108	129	136	137	140	152	171	186	189	207	232	240	262	306	307	311	322	363	389	393	413	417	486
Indiv.																									
1	1	1	0	0	1	1	0	1	0	0	1	0	0	1	0	1	0	1	1	1	0	1	1	1	0
2	1	0	1	0	1	0	0	1	0	0	1	1	0	1	1	0	0	0	1	0	1	0	0	1	0
3 east	0	1	1	0	0	1	0	0	1	1	0	1	1	1	0	1	1	0	1	0	1	0	1	1	0
3 west	0	1	1	0	0	1	0	0	1	1	0	1	1	1	0	1	1	0	1	0	1	0	1	1	0
4	1	0	1	1	1	0	0	1	0	0	0	1	1	1	1	1	0	0	1	0	1	0	0	1	0
5	1	1	1	1	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	1
6	1	1	1	0	1	0	0	1	1	0	0	1	1	1	0	1	0	0	1	1	1	0	1	1	1
7 female	1	1	1	1	1	0	1	1	0	0	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0
7 male	1	1	1	0	1	0	0	1	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0	1	0
8	0	1	1	0	1	0	0	1	0	0	1	1	0	1	0	1	1	0	1	0	1	0	1	1	1

(AFLP *cac* primer: 92 monomorphic peaks not shown).

Size	105	107	108	119	123	124	135	154	167	168	183	185	186	190	194	197	198	200	205	206	210	210	238	244
Indiv.																								
1	0	0	0	0	1	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	0	1	0	0
2	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	1	1	1	0	1	0
3 east	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1
3 west	0	0	0	1	1	0	0	1	0	0	0	1	1	0	0	0	1	0	0	1	0	1	0	0
4	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	1	1	0	1	0
5	1	1	1	1	1	0	0	1	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	0
6	1	1	1	1	0	1	1	1	1	0	0	0	0	0	1	1	0	1	0	0	1	1	1	1
7 female	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0
7 male	0	0	0	1	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	1	1	0	0	0
8	0	0	0	0	1	0	0	1	0	1	0	0	1	1	1	0	0	0	0	1	0	1	0	1

Size	245	247	254	256	261	269	276	280	284	322	326	388	392	394	426
Indiv.															
1	1	0	0	1	0	0	0	1	0	0	1	1	1	1	0
2	0	1	0	1	0	1	1	1	0	0	0	1	1	0	1
3 east	1	0	0	0	1	1	1	1	1	0	0	1	0	0	1
3 west	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1
4	1	0	0	1	0	0	1	1	0	0	0	0	1	1	0
5	1	0	0	0	0	1	1	1	0	0	0	0	1	0	0
6	1	0	1	0	1	1	1	1	0	1	0	0	0	0	0
7 female	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0
7 male	1	0	0	1	0	0	1	1	0	0	0	0	1	0	0
8	1	1	0	0	0	0	0	1	1	0	0	0	1	0	0

CANADIAN HEMLOCK (AFLP *caa* and *cac* primers: 44 monomorphic peaks not shown).

Primer	caa	cac	cac	cac	cac	cac	cac
Size	141	280	282	287	290	293	296
Indiv.							
1	0	1	0	1	0	1	0
2	0	0	1	0	1	0	1
3	0	0	1	0	1	0	1
4	0	1	0	1	0	1	0
5	1	--	--	--	--	--	--

TULIP POPLAR

(AFLP *caa* primers: 70 monomorphic peaks not shown).

Size	100	140	141	155	163	164	186	196	207	208	210	212	232	243	263	304	311	317	320	326	356	357	452	456	460
Individual:																									
1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	0	0	1	0	1	1	0	1	1	1
2	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0
3	1	0	0	1	0	1	0	0	1	1	0	1	1	1	1	0	0	1	0	0	1	0	1	1	1
4	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0	1	1	1
5	1	1	0	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	0	1	1	0	1	1	1
6	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	0	1	0	1	1	0	1	1	1
7	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	0	0	0	1	1	0	0	0	0	0
Monticello	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	1	1	1	1

(AFLP *cac* primers: 36 monomorphic peaks not shown).

size	110	115	116	119	120	121	123	127	134	135	143	147	150	154	156	166	172	179	184	190	196	212	217	218
Individual:																								
1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
2	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	1	0	0	1	1
3	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
4	1	1	1	1	0	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0
5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
6	--	--	--	--	--	0	--	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	1
Monticello	1	1	1	1	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	0	1

size	226	238	240	241	242	244	246	254	257	259	260	262	274	276	280	285	290	311	314	379	381	392	394
Individual:																							
1	0	0	0	0	1	0	0	1	0	0	1	0	1	1	0	1	0	1	1	0	1	0	1
2	1	1	1	0	0	1	1	1	0	0	1	0	1	1	1	1	0	1	0	1	0	0	0
3	1	0	0	0	1	0	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	1
4	1	1	1	0	0	1	0	1	0	0	0	1	0	1	1	0	1	0	1	0	1	1	0
5	1	0	0	0	1	0	0	0	1	1	1	0	1	1	0	1	0	1	1	0	1	0	1
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	1	1	0	0	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	1
Monticello	1	1	1	0	1	1	0	0	0	1	1	0	1	1	0	1	1	0	1	1	1	0	1

WHITE ASH

(AFLP *caa* and *cac* primers: 154 monomorphic bands not shown).

	caa	caa	caa	caa	cac	cac	cac	cac	cac	cac	cac	cac	cac	cac	cac	cac	cac	cac	cac	cac	cac	cac	cac
Size	187	209	249	301	100	101	105	118	149	150	151	160	161	162	193	210	218	263	264	267	269	330	
Indiv.																							
1	--	--	--	--	--	--	--	--	--	--	1	--	--	--	--	--	--	--	--	--	--	--	
2	0	0	1	1	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	
3	0	1	1	1	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	1	0	1	
4	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	
5	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	
6	0	1	0	1	1	1	0	0	1	1	1	0	0	1	0	0	1	1	1	0	1	0	

WHITE MULBERRY

(AFLP *caa* and *cac* primers: 8 monomorphic bands not shown).

	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	caa	cac
Size	121	128	130	142	148	158	161	164	197	213	216	228	250	266	271	389	424	350				
Individual:																						
Mount Vernon	1	1	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1				
Monticello	0	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	1	0				

APPENDICES

a. **Laboratory Techniques**
b. **Explanation of Genetic Concepts**
c. **References**

Appendix a: LABORATORY TECHNIQUES

i. Isozymes
ii. DNA Extraction
iii. RAPDs
iv. AFLPs

i. ISOZYMES

Tissue preparation. The plant material processed varied by species (Methods: Table 1). The material was ground in a mortar using liquid nitrogen. Approximately 0.4 ml of a simple Tris buffer, pH 7.5 (Gottlieb 1981) or a more complex Tris buffer, pH 8.0, with sucrose (USDA Forest Service 2003) was added to the ground powder and mixed. As the resulting tissue mass thawed, 120 µl/sample of slurry was transferred to a microtiter plate well, and a total of three replicate plates were made. Plates were stored at -70°C. On the morning of electrophoresis, the samples were thawed and soaked onto 3mm wide wicks made of Whatman 3MM chromatography paper.

Electrophoresis. Methods of sample preparation and electrophoresis follow the general methodology of Conkle et al. (1982), with some modifications (USDA Forest Service 2003). All enzymes were resolved on 11% starch gels. Four run buffers were used: a lithium borate electrode buffer (pH 8.3) was used with a Tris citrate gel buffer (pH 8.3) (Conkle et al. 1982), a sodium borate electrode buffer (pH 8.0) was used with a Tris citrate gel buffer (pH 8.8) (Conkle et al. 1982), and two morpholine citrate electrode and gel buffers (pH 6 and pH8) (USDA Forest Service 2003) to resolve a total of 18 enzymes (Methods: Table 2). Enzymes assayed in all species included: aspartate aminotransferase (AAT), aconitate hydratase; aconitase (ACO); alcohol dehydrogenase (ADH); diaphorase (DIA); flourescent esterase (FEST); glucose-6-phosphate dehydrogenase (G6PD); glutamate dehydrogenase (GDH); glycerate-2-dehydrogenase (GLYDH); isocitrate dehydrogenase (IDH); leucine aminopeptidase (LAP); malic acid dehydrogenase (MDH); malic enzyme (ME); 6-phosphogluconate dehydrogenase (6PGD); phosphoglucoisomerase (PGI); phosphoglucomutase (PGM); shikimate dehydrogenase (SKD); superoxide dismutase (SOD); triose phosphate isomerase (TPI); uridine diphosphoglucose pyrophosphorylase (UGPP). SOD was scored on GDH gels.

Gels were photographed and scored from the photographs. Genetic interpretations were inferred directly from isozyme phenotypes based on knowledge of the generally conserved enzyme substructure, compartmentalization, and isozyme number in higher plants (Gottlieb 1981, 1982; Weeden and Wendel 1989). The number of enzymes resolved and the number of loci per enzyme varied from species to species (Methods: Table 3). American Holly is tetraploid, White Ash can be diploid, tetraploid, or hexaploid, and the other species studied are diploid (Darlington and Wylie 1955). Due to lack of variation, all species were treated as diploid for analysis.

Results were analyzed using Popgene version 1.21 (Yeh et al. 1997). A locus was considered polymorphic if an alternate allele occurred even once. Statistics calculated included unbiased genetic distance (Nei 1978), effective number of alleles per locus (Kimura and Crow 1964), expected heterozygosity (Nei 1973), and gene flow (Slatkin and Barton 1989).

Methods: Table 1. Tissue preparation for isozyme analysis of Washington trees. Grinding buffers: G = simple Tris buffer, pH 7.5 (Gottlieb 1981); M/N = a more complex Tris buffer, pH 8.0, with sucrose (USDA Forest Service 2003). lq N = liquid nitrogen.

Species	Tissue Type	Tissue Quantity	G buffer	M/N buffer	lq N used
American Holly	leaves	8 mm^2	X	X	yes
Canadian Hemlock	leaves	8 mm^2	X	X	yes
Tulip Poplar	dormant buds	3 mm^3	X	X	no
White Ash	dormant buds	3 mm^3	X	X	no
White Mulberry	dormant buds	3 mm^3	X	X	no
Tulip Poplar	leaves	8 mm^2		X	yes
White Ash	leaves	8 mm^2		X	yes
White Mulberry	leaves	8 mm^2		X	yes

Methods: Table 2. Buffer / enzyme combinations used for isozyme analysis of Washington trees. Buffer abbreviations: LB = lithium borate pH8.3, SB = sodium borate pH 8, MC6 = morpholine pH6, and MC8 = morpholine pH8. See text of 'Laboratory Techniques: Isozymes' for enzyme abbreviations.

LB buffer	SB buffer	MC6 buffer	MC8 buffer
ACO	AAT	DIA	DIA
ADH	GDH	FEST	FEST
FEST	G6PD	IDH	IDH
IDH	GLYDH	MDH	MDH
LAP	6PGD	6PGD	6PGD
ME	TPI	SKD	SKD
PGI	UGPP		
PGM			

Methods: Table 3. Number of loci resolved for each enzyme in five species of tree. See text of 'Laboratory Techniques: Isozymes' for enzyme abbreviations. --- = not resolved in this species. * = all individuals heterozygous.

Enzyme	American Holly	Canadian Hemlock	Tulip Poplar	White Ash	White Mulberry
AAT	1	1	2	---	1
ACO	---	---	1	---	---
ADH	1	---	1*	---	1
DIA	1*	1	1	1	1
FEST	1	---	1	1	1
G6PD	---	1	1	---	---
GDH	---	1	1	---	1
IDH	1	1	1	1	1
LAP	1	1	---	---	1
MDH	3	3	3	2	2
ME	1	1	1	---	1
6PGD	---	3	2	---	3
PGI	1	2	2	---	2
PGM	---	2	1	---	1
SKD	---	1	1	---	1
TPI	2*	1	2	2	2
UGPP	---	1	1	---	1

ii. DNA EXTRACTION

DNA was extracted from plant leaf tissue from the five Washington tree species using three different protocols: (1) DNeasy Plant Mini Kit (Qiagen 2000), (2) Dneasy Plant Maxi Kit (Qiagen 2000), and (3) Fast-Prep protocol (Qbiogene). Protocols were assessed for quality of DNA produced by running samples on 0.8%, 1X TBE, agarose gels stained with ethidium bromide. DNA yields were higher with DNeasy Plant Mini and Maxi kits except for White Mulberry (Methods: Table 4). Sample DNA used in analysis was extracted using the DNeasy Plant kits with the exception of sample the White Mulberry sample from Monticello, which was prepared using the Fast Prep Protocol.

Methods: Table 4. Concentrations (in nanograms per microliter) of DNA extractions for Washington Trees. D = DNeasy kit (Qiagen 2000). F = Fast Prep kit (QBiogene). M=Mini kit; X=Maxi kit. All extraction dates in 2002.

Species	Individual	Method:	D	F	D	D	D	D	D	F
		Version:	(M)		(M)	(X)	(X)	(X)	(X)	
		Date:	4/3	4/5	4/26	5/16	5/20	5/21	6/3	6/4
American Holly	1		21	103	34					
American Holly	2		20	99	25					
American Holly	3E		48	103	26					
American Holly	3W		41	110	32					
American Holly	4		30	112	26					
American Holly	5		38	119	18					
American Holly	6		29	126	32					
American Holly	7F		26	110	34					
American Holly	7M		24	82	32					
American Holly	8		56	157	19					
Canadian Hemlock	1		58	181	109					
Canadian Hemlock	2		55	178	124					
Canadian Hemlock	3		74	249	171					
Canadian Hemlock	4		172	200	120					
Canadian Hemlock	5		63	148	78					
Tulip Poplar	TP1					42				
Tulip Poplar	TP2					45				
Tulip Poplar	3						51			
Tulip Poplar	4						76			
Tulip Poplar	5						41			
Tulip Poplar	6							34		
Tulip Poplar	7						67			
Tulip Poplar	Monticello								24	
White Ash	WA1					93				
White Ash	WA2					42				
White Ash	3							63		
White Ash	4							47		
White Ash	5							36		
White Ash	6								28	
White Mulberry	1								16	112
White Mulberry	Monticello								6	41

iii. RAPDs

American Holly and Canadian Hemlock were screened with 16 primers per species: OPK1 - OPK16 (Operon Technologies, Alameda, California). Tulip Poplar, White Ash, and White Mulberry were screened with 12 primers per species: OPK1 - OPK8, OPK17 - OPK20. For each sample 3 nanograms (6.0 µl) DNA was combined with 11.3 µl ddH2O, 4.0 µl dNTPs, 2.5 µl 10X buffer, 0.2 µl (0.2 U) *Taq* polymerase, and 1.0 µl (20 pmol) primers in a final volume of 25 ml. PCR reactions were carried out in a MJ Research PT-100 thermal cycler using conditions outlined in Methods: Table 5. The product was visualized on 1.4% agarose gels stained with ethidium bromide.

Methods: Table 5. PCR conditions for RAPD analysis of Washington Trees.

Step	Temp.	Time	Temp.	Time	Temp.	Time
1	94°C	1:30 min				
2-40	94°C	1:00 min	40°C	1:00 min	72°C	2:00 min
41	72°C	10:00 min				
42	4°C	until removed from machine				

iv. AFLPs

DNA fingerprinting was conducted using the AFLP technique according to the methods of Vos et al. (1995), as modified (Applied Biosystems 2000), except that two labeled *Eco*RI selective amplification primers were tested, a fluorescent 6-FAM (6-carboxyfluorescein) label and a HEX (6-carboxy-1,4-dichloro-2',4',5',7'-tetra-chlorofluorescein) label on the 5' nucleotide.

DNA was digested using restriction endonucleases *Eco*RI and *Mse*I. Each sample was incubated at 37°C for two hours in the following solution: 1.0 µl 10X T4 ligase buffer, 1.0 µl 0.5 M NaCl, 0.5 µl BSA (1 mg/µl), 2.0 µl *Eco/Mse* Adapter mix, 0.3 µl ddH2O, and 1.0 µl master mix. (Master mix for 10 samples = 1.0 µl 10X T4 ligase buffer, 1.0 µl 0.5 M NaCl, 0.5 µl BSA (1 mg/µl), 1.0 µl *Mse*I (10,000 units/ml), 2.5 µl *Eco*RI (20,000 units/ml), 0.4 µl T4 DNA ligase (670 NEBU), and 3.6 µl ddH2O.) The amount of DNA used in each reaction was kept constant within species but varied among species; 0.1 µg DNA for American Holly, 0.3 µg DNA for Canadian Hemlock, 0.19 µg DNA for Tulip Poplar, 0.13 µg DNA for White Ash, and 0.1 µg DNA for White Mulberry.

For preselective amplification, 4.0 µl of the restriction ligation product was transferred to a new 0.2 ml tube with 1.0 µl *Mse*I + *Eco*RI preselective primer and 15.0 µl AFLP core mix. Amplification was performed by PCR (Methods: Table 6). Half the product was visualized on 1.5% agarose gels stained with ethidium bromide to verify the success of the PCR, and the other half was diluted with 190 µl 0.1X TE buffer and used for selective amplification.

Selective amplifications were performed using four *Eco*RI +3 / *Mse*I +3 primer pairs (e.g. E.ACT//M.CAA, E.ACT//M.CAC, E.AAG//M.CTG, E.AAG//M.CTC), where E and M designate the *Eco*RI and *Mse*I adapters with three selective nucleotides as described by Vos et al. (1995). The E.ACT primer was labeled with 6-FAM and the E.AAG was labeled with HEX. Each reaction used 3.0 µl diluted product from preselective amplification, 1.0 µl *Mse*I primer [Cxx] at 5 µM concentration, 1.0 µl *Eco*RI primer [Axx] at 1 µM concentration, and 15.0 µl

AFLP core mix (Applied Biosystems 2000). Amplification was performed by PCR (Methods: Table 7).

The amplified DNA fragments were size fractionated using an ABI 3100 instrument with 36-cm capillaries, POP-4 polymer, Genescan 400HD [ROX (rhodamine X)] internal size standard, and Genescan software (PE Applied Biosystems, Foster City, CA). Each sample consisted of 0.5 μl of selective amplification product, 10 μl formamide and 0.02 μl internal size standard.

The GeneScan sample files were analyzed for the presence and absence of DNA fragments between 50 and 400 bp in length using ABI PRISM Genotyper® 3.7 NT Software. Results of this analysis were rescored manually by two observers.

Methods: Table 6. PCR conditions for the preamplification step of AFLP analysis of Washington Trees.

Step	Temp.	Time	Temp.	Time	Temp.	Time
1	72°C	2:05 min				
2 – 20	94°C	0:20 min	56°C	0:30 min	72°C	2:00 min
21	60°C	30:00 min				
22	4°C	until removed from machine				

Methods: Table 7. PCR conditions for the selective amplification step of AFLP analysis of Washington Trees.

Step	Temp.	Time	Temp.	Time	Temp.	Time
1	94°C	2:00 min				
2	94°C	0:20 min	66°C	0:30 min	72°C	2:00 min
3	94°C	0:20 min	65°C	0:30 min	72°C	2:00 min
4	94°C	0:20 min	64°C	0:30 min	72°C	2:00 min
5	94°C	0:20 min	63°C	0:30 min	72°C	2:00 min
6	94°C	0:20 min	62°C	0:30 min	72°C	2:00 min
7	94°C	0:20 min	61°C	0:30 min	72°C	2:00 min
8	94°C	0:20 min	60°C	0:30 min	72°C	2:00 min
9	94°C	0:20 min	59°C	0:30 min	72°C	2:00 min
10	94°C	0:20 min	58°C	0:30 min	72°C	2:00 min
11	94°C	0:20 min	57°C	0:30 min	72°C	2:00 min
12	94°C	0:20 min	56°C	0:30 min	72°C	2:00 min
13	60°C	30:00 min				
14	4°C	until removed from machine				

Appendix b: EXPLANATION OF GENETIC CONCEPTS

i. Gene Basics
ii. Gene Terminology
iii. DNA fingerprinting
iv. PCR
v. Isozymes
vi. RAPDs
vii. AFLPs
viii. Polyploidy
ix. Statistics for Population Genetics

i. GENE BASICS

A gene is a region on a molecule of the chemical DNA. Genes are written on DNA in an alphabet made up of four chemicals we abbreviate as A, C, G, and T. In general, each gene contains the instructions, the code, for ultimately making a protein. A stretch of DNA that will make a protein is called a 'coding region'. However, many long stretches of DNA do not code for proteins. These areas of DNA are called 'non-coding regions'.

Genetic variation is differences in the genetic code in two or more DNA molecules. Protein-coding regions are much less variable than non-coding regions. That makes sense because the coding regions must produce functional proteins. The non-coding regions have few restrictions in their code. Some genetic markers (DNA fingerprinting methods) look at protein coding regions, but most look at highly variable non-coding regions.

Proteins have many roles in an organism. Some important proteins are enzymes, proteins that control chemical reactions. Isozymes measure the genes that produce these enzymes.

ii. GENE TERMINOLOGY

We all know more or less what we mean by the word "gene," but the word "gene" covers two or more different, related ideas. A locus is a place on DNA. It is a place in the sense that the upper right hand corner of a one-dollar bill is a place; it is the same place on every comparable dollar bill. The locus for, say, the enzyme catalase is located in the same place in the DNA in each individual of a given species. At the catalase place on the DNA, every organism has a code for making the enzyme catalase (or a messed up version of it).

An allele is a version of the code found at a locus. At the catalase locus, three individuals may have slightly different DNA codes that make different forms of catalase; between them they have three different alleles for catalase. The more alleles found at a locus, the more genetic variation is contained in the DNA.

iii. PCR

The polymerase chain reaction (PCR) allows the researcher to make many copies of a chosen region of DNA, which has revolutionized the study of genetics. In itself, PCR tells us nothing about DNA, but it is essential because any procedure that will answer our questions requires thousands of copies of the DNA.

In the living cell, copying DNA is a complex process requiring many enzymes to work together. Some enzymes untwist the coiled DNA, others separate the two complementary DNA strands (a difficult task), the enzyme DNA polymerase makes copies, and other enzymes speed raw materials to the work site.

The PCR process amplifies (makes copies of) DNA at an astonishing rate. The ingredients for PCR are DNA, special DNA polymerases that work at high temperatures, primers (chemicals that tell the polymerases what part of the DNA to copy), and the buffers needed to keep the chemicals stable. The ingredients are put in a machine that makes the solution hot, so the DNA strands separate, then cools it down so the primers can attach to the DNA, then warms it up a bit so the polymerase can work at a reasonably fast rate, and then raises the temperature again to separate the DNA strands, cools again, etc. If all goes well, the amount of the desired DNA might nearly double with each cycle. The basic procedure is the same for all PCR reactions, and the product formed depends on the primers, which define the regions of DNA that will be copied.

iv. DNA FINGERPRINTING

DNA Fingerprinting is any method of identifying individuals by traits of their DNA. "Genetic Profiling" is another term often used in place of "Genetic Fingerprinting". Some procedures start with a lot of DNA and cut it into pieces. Others start with tiny amounts of DNA and amplify (make copies of) selected pieces using PCR. Nearly all DNA fingerprinting methods then sort the pieces by size, stain the DNA so it can be seen, and score what size DNA pieces are present. The results are photographed. The photograph shows a series of bright bands or lines. Each band is a cluster of DNA pieces of the same size.

All DNA fingerprinting methods are, to a greater or lesser extent, complicated by questions of interpretation. Should a very faint band be counted as present or absent? Are two bands that traveled almost the same distance the same or not? Do differences in band intensity encode a genetic difference that should be counted, or simply differences in technique? With good equipment and experienced technicians, these problems are minimized.

An important principle of DNA fingerprinting is that exclusion is absolute, but inclusion is tentative. In other words, if DNA fingerprints from two samples fail to match, we can be sure that they come from different individuals. However, if DNA fingerprints do match, we cannot be completely sure that they come from the same individual. We can believe they are the same with a certain level of probability. If the markers used are variable and the species is well studied, we may be more than 99.9999% sure that two samples with matching DNA fingerprints came from the same individual. If the markers show little variation or if the species is poorly studied, we have much less certainty.

v. ISOZYME MARKERS

Isozyme analysis evaluates variation in DNA indirectly, by assessing variation in proteins. (DNA carries the instructions for making proteins, so differences among proteins must result from differences in DNA.) The proteins studied in isozyme analysis of plants are enzymes, proteins that control chemical reactions. Each different kind of enzyme controls a different reaction, using different raw materials (substrates).

How: Plant material is ground in a buffer that permits the enzymes to retain their normal, functional shape. The plant extract is placed on the edge of a slab of starch gel. An electric current is applied to the gel. Because proteins have small electric charges, they move through the gel in response to the current. Enzymes with different charges move at different speeds. When the different proteins have been sufficiently separated, the gel is removed from the current. The researcher supplies an appropriate substrate for the enzyme of interest, plus chemicals that will change color when the enzyme has changed the substrate. The location of each patch of color on the gel shows where the enzyme migrated. Different locations are scored as different alleles.

Reasons to use isozymes: Isozyme analysis of the most commonly used enzymes reveals moderate levels of variation. It is very good for measuring variability within and among populations of a single species or a group of closely related species, in part because results can be compared to large databases of previous information (Gitzendanner and Soltis 2000, Godt and Hamrick 1998, Hamrick and Godt 1990) so we know what the results mean. Depending on the amount of variation in the population and the number of enzymes examined, isozyme analysis may be useful for identifying clones and for paternity analysis. The procedure reveals both alleles each individual carries (the copy from its mother and the copy from its father) and is relatively inexpensive.

Reasons not to use isozymes: Results of isozyme analysis are influenced by small differences in lab techniques, so results from different labs are often not comparable. Isozyme analysis tests only a few DNA sequences. All of them code for essential proteins, so changes are often so harmful that the individual dies. As a result, isozymes show little variation compared to non-coding sequences. Some species or populations have too little variation in protein structure for this method to be useful for clone identification or paternity analysis.

vi. RAPD MARKERS

RAPDs are Randomly Amplified Polymorphic DNA. Certain sections of DNA are amplified (copied), without anyone knowing quite what those sections are. This method assesses variation in many parts of the DNA, some of them regions that change quickly and others that change slowly.

How: PCR is performed with a short, arbitrarily chosen primer. The primers cause the amplification of certain sections of DNA – we can't predict much about those sections. After PCR, the DNA is placed on a gel and an electric current is applied. The DNA moves through the gel and small pieces move faster than large ones. Therefore, the pieces become sorted by size. The DNA is stained, and individuals are scored by the presence or absence of DNA of particular sizes. If the particular DNA section amplified has different lengths in different individuals, those differences will show up on the gel. Additional variation is produced if some individuals have a slightly different DNA sequence that cannot bind to the primer at the sites; these individuals will lack a band and be scored with a '0' to indicate 'band absence'.

Reasons to use RAPDs: This technique reveals high levels of variation, and therefore is well suited for identifying individuals or clones, and for paternity analysis. It can also be used to assess genetic variation in populations. This technique samples DNA randomly, and therefore assesses variation in both protein coding and non-coding regions.

Reasons not to use RAPDs: The technique is sensitive to the quality of DNA used and so cannot be used for degraded DNA. It is also sensitive to variation in PCR technique; results may be inconsistent. The genetics of the DNA markers used are usually unknown; results of paternity analysis are sometimes unexpected. The technique does not consistently reveal both alleles that an individual carries (the allele from its mother and the allele from its father).

vii. AFLP MARKERS

AFLPs are Amplified Fragment Length Polymorphisms – copies of DNA fragments of different lengths. Like RAPDs, this technique tests variation in many DNA sections, some of which change quickly and others change slowly.

How: DNA is cut into fragments with special enzymes that cut at specific DNA sequences. The cut ends are covered with short primers. PCR is used to make many copies of the fragments. (In practice, only the shorter fragments, those less than approximately 3,500 "letters" long, can be copied in PCR.) PCR produces more copies than can be studied easily, so a second PCR is performed, using longer primers that permit copying of only a few of the fragments. After PCR, the DNA is placed on a gel and an electric current is applied. The DNA moves through the gel and small pieces move faster than large ones. Therefore, the pieces become sorted by size. The DNA is stained, and individuals are scored by the presence or absence of DNA of particular sizes. If a particular DNA section amplified has different lengths in different individuals, those differences will show up.

Reasons to use AFLPs: This technique reveals high levels of variation, and therefore is well suited for identifying individuals or clones, and for paternity analysis. It can also be used to assess genetic variation in populations. Like RAPDs, this technique samples many regions of the DNA, some of which change quickly and others slowly. This technique is less sensitive to the quality of DNA used than RAPDs, and results are more consistent. Sometimes both alleles at a locus can be detected. AFLPs sample mainly non-coding regions, and multiple repeats.

Reasons not to use AFLPs: The technique is more complex and difficult than RAPDs, and more expensive. Often, the technique cannot reveal both alleles that an individual carries.

viii. POLYPLOIDY

Most organisms have two sets of chromosomes (a long DNA molecule, packaged in protective proteins) in every cell (except sperm and egg cells). In other words, most organisms are diploid. One set of chromosomes came from the mother, and one from the father. With minor exceptions, each set of chromosomes has the same kind of information. For example, if one chromosome in one set has a gene for hair color, the corresponding chromosome in the other set also has a gene for hair color, in the same place (at the same locus).

Humans and most vertebrates are extremely intolerant of abnormal chromosome numbers. A single excess chromosome may cause a serious disorder like Down's syndrome or, more often, prevent the embryo from surviving to term. Having a whole extra set is always fatal, usually long before birth.

Plants are much more tolerant of excess chromosomes. In fact, many plants normally have four sets (are tetraploid) or more. For example, different cultivated strains of the lawn grass Red Fescue typically has six or eight sets in each cell. This seems to work well for plants. Organisms that have more than two sets of chromosomes per cell are polyploid.

Polyploidy can be found in diverse plants in any habitat, but it is especially common in artic and alpine species. It may allow the plant to produce adequate amounts of protein at a reasonably fast rate despite the slow rate of chemical reactions in cold weather.

ix. STATISTICS FOR POPULATION GENETICS

Genetic diversity of populations is measured in several different ways. Frequently used statistics are explained below.

P = percent polymorphic loci. Of the loci tested, what percentage are polymorphic. A locus is considered polymorphic if two or more alleles are detected at that locus.

A = alleles per locus. How many alleles are observed at each locus, on average.

Ae = effective alleles/locus. This is a measure of how common the alleles are at each locus. If the population has two alleles at a locus and they are equally common, the alleles are equally effective, and *Ae* = 2.0. If the one allele occurs in over 99% of individuals and the other is rare, *Ae* is much lower, barely above 1.0.

Ho = observed heterozygosity. At a locus, individuals may be homozygous (have the same alleles) with genotypes AA or BB, for example. Individuals with the mixed genotype AB are considered heterozygous. Observed heterozygosity is the proportion of the individuals that are heterozygous.

He = expected heterozygosity. This is the proportion of individuals that "should" be heterozygous, calculated from the observed frequency of each allele. In itself, expected heterozygosity, like Ae, tells something about the relative abundance of alleles. *Ho* becomes much more useful when compared with *He*. Then it tells something about breeding system or

selective pressures. If *Ho* is much less than *He*, the population is probably somewhat inbred. In plants, this is often a result of self-pollination. If *Ho* is greater than *He*, natural selection may be favoring heterozygotes (which often show hybrid vigor), or the organisms mate mainly with others unlike themselves, or the plant may be a polyploid that is being measured as a diploid.

H = genetic diversity. Like *Ho* and *He*, this statistic tells about the relative abundance of alleles. It is much less specific than *Ho* or *He*. Now it is used mainly when the markers are dominant markers, which don't permit measuring *Ho*, or for comparison with other studies in which *H* was calculated.

F = inbreeding coefficient. $F = (He-Ho)/He$. When *F* is positive, a population is somewhat inbred. In plants, that is often due to pollination of some seeds by pollen from the same individual plant. A positive *F* can also result from inbreeding in a very small population. If *F* is negative, the plant is outbred and something has favored heterozygotes more than homozygotes. Heterozygous plants often show hybrid vigor that gives them a better chance of survival, so in general a sample of older plants would be expected to have a more negative *F* than a population of seedlings.

Genetic identity (similarity) or genetic distance. These are measures of how similar (or different) populations are, based on the frequency of each allele in each population. In general, populations of the same species (and subspecies) have identities greater than 0.9, on a scale of 0 to 1.0. Populations of different subspecies of the same species usually have lower genetic identities, perhaps 0.8. Populations of different closely related species have genetic identities averaging around 0.68, though they may be greater than 0.9 or less than 0.3. Calculating genetic identities of very distantly related species violates the assumptions of the equation, creating unreliable values.

Fst and *Gst* are measures of the differentiation among populations. They are particularly useful for determining where most differentiation lies, in a hierarchy of individuals within subpopulations, and subpopulations within populations. On a scale of 1 to 1.0, *Fst* values of 0 – 0.05 show little genetic differentiation; 0.05 – 0.15 shows moderate differentiation; 0.15 to 0.25 shows great differentiation, and values above 0.25 show very great differentiation.

Appendix c: REFERENCES

Applied Biosystems. 2000. AFLP™ Plant Mapping Protocol. Applied Biosystems, Foster City, California, available through http://www.appliedbiosystems.com.

Conkle, M. T., Hodgskiss, P. D., Nunnally, L. B., and S. C. Hunter. 1982. Starch Gel Electrophoresis of Conifer Seeds: a Laboratory Manual. Gen. Tech. Rep. PSW-64. Berkeley, CA: Pacific Southwest Forest and Range Experiment Station, Forest Service, U.S. Department of Agriculture. 18pp.

Darlington, C. D. and A. P. Wylie. 1955. Chromosome Atlas of Flowering Plants. 2nd ed. George Allen and Unwin Ltd., London, pp. 139.

Gitzendanner, M. A. and P. S. Soltis. 2000. Patterns of genetic variation in rare and widespread plant congeners. American Journal of Botany 87:783-792.

Godt, M. J. and Hamrick, J. L. 1998. Allozyme diversity in the grasses. pp. 11 – 29 *in* Cheplick, G. P., editor. Population Biology of Grasses. Cambridge University Press. Cambridge, England.

Gottlieb, L. D. 1981. Electrophoretic evidence and plant populations. Prog. Phytochem. 7: 1-46.

Gottlieb, L. D. 1982. Conservation and duplication of isozymes in plants. Science 216: 373-380.

Hamrick, J. L. and M. J. W. Godt. 1990. Allozyme diversity in plant species. pp. 43 – 63 *in:* Brown, A. H. D., M. T. Clegg, A. L. Kahler, and B. S. Weir (eds.). Plant Population Genetics, Breeding, and Genetic Resources. Sinauer Associates, Inc., Sunderland, Massachusetts.

Kimura, M. and J. F. Crow. 1964. The number of alleles that can be maintained in a finite population. Genetics 49: 725-738.

Nei, M. 1973. Analysis of gene diversity in subdivided Populations. Proc. Nat. Acad. Sci. USA 70(12): 3321-3323

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Qiagen. 2000. DNeasy Plant Mini Kit and DNeasy Plant Maxi Kit Handbook for DNA Isolation from Plant Tissue. Qiagen, Inc., Valencia, California, available at http://www1.qiagen.com/literature/handbooks/.

Qbiogene. BIO 101 Protocol: FastDNA Kit. Catalog # 6540-400. QBiogene, Vista, California. See http://222.qbiogene.com

Shannon, C. E. and W. Weaver. 1949. The mathematical theory of communication. Urbana IL: University of Illinois Press.

Slatkin, M. and Barton, N. H. 1989. A comparison of three indirect methods for estimating average levels of gene flow. Evolution 43: 1349-1368.

USDA Forest Service. 2003. National Forest Genetic Electrophoresis Laboratory Standard Operating Procedures. NFGEL, USDA Forest Service, Placerville, California.

Vos, P. R., Hogers, M., Bleeker, M., Reijans, T., Vande Lee, T., Hornes, M., Vrijters, L., Pot, J., Peleman, M., Kuiper, M., and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucl. Acids Res. 23: 4407 – 4414.

Weeden, N. F. and J.F. Wendel. 1989. Genetics of plant isozymes. pp. 46-72 *in* Isozymes in Plant Biology, eds. D. E. Soltis and P. S. Soltis. Dioscorides Press, Portland, Oregon.

Yeh, F. C., Yang, R.-C., and T. Boyle. 1997. Popgene Version 1.20. University of Alberta, Edmonton, Alberta.

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Final Report

Genetic Affinities of a Wallflower (*Erysimum*) Population from Lake Earl Dunes in Coastal Northern California

Erysimum menziesii © Ken Berg and CNPS

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MANAGEMENT SUMMARY

Management Question

The wallflower population at Lake Earl Dunes resembles both *Erysimum menziesii,* which is listed as rare under the federal and California Endangered Species Acts, and *E. concinnum,* which is rare but not listed. This Lake Earl population is currently classified as *E. concinnum*, but its taxonomic identity is in doubt.

Study Methods

A laboratory genetic analysis (isozymes) was used to determine the taxonomic identity of the Lake Earl Dunes wallflower population. The Lake Earl Dunes population was compared with a nearby *Erysimum concinnum* population, and one population each of *E. menziesii* ssp. *eurekense, E. menziesii* ssp. *menziesii,* and *E. menziesii* ssp. *yadonii*.

Study Results

In its isozyme patterns, the Lake Earl Dune population most closely resembles the *Erysimum concinnum* population. There is some genetic similarity between the Lake Earl Dunes population and *E. menziesii* ssp. *menziesii*, perhaps indicating some past hybridization or introgression between the Lake Earl Dunes plants and those of *E. menziesii* ssp. *menziesii*. The *E. menziesii* ssp. *eurekense* population is very distinct genetically from any other of the populations studied. The two most dissimilar populations are the *E. concinnum* and *E. menzies*ii ssp. *eurekense* populations. The Lake Earl Dunes population is the most genetically diverse population, while the *E. menziesii* ssp. *eurekense* population contains the least amount of genetic variation in the study.

Management Implications

This isozyme study indicates that the Lake Earl Dune population is more similar to *E. concinnum* than it is to *E. menziesii*. Of the three *E. menziesii* subspecies, the Lake Earl Dunes population does share some genetic similarity with *E. menziesii* ssp. *menziesii* showing some potential hybridization or introgression occurring in this population. Morphologically, the Lake Earl Dune population resembles *E. concinnum* in fruit orientation, but *E. menziesii* in height, color, hairiness, flower shape, and habitat. All of these traits are considered important for classifying *Erysimum* species.

INTRODUCTION

Confusing arrays of beautiful yellow- or white-flowered wallflowers (*Erysimum*; Brassicaceae) inhabit sand dunes and coastal headlands of northern California and southern Oregon. These plants are considered members of the *Erysimum capitatum* alliance. In California, the widespread *E. capitatum* has apparently produced many descendent species with limited range, most of them associated with particular substrates.

A unique wallflower population grows in the Lake Earl Dunes near Crescent City, California. It is somewhat intermediate between two members of the alliance, *Erysimum concinnum* and *E. menziesii* which has been divided into three subspecies (Table 1). Based largely on a single morphological characteristic, fruit orientation, this population has been classified as *Erysimum concinnum* (Price 1987). However, the Lake Earl population resembles *E. menziesii* ssp. *eurekense* in stature, color, pubescence, and flower shape, and these traits remain consistent when the plants are grown in the greenhouse (David Imper, pers. comm.). In addition, the Lake Earl population lives in a dune mat habitat typical of *E. menziesii*, not the coastal bluffs and slopes typical of *E. concinnum.*

Table 1. Morphological traits distinguishing *Erysimum concinnum* and three subspecies of *E. menziesii*.

Trait	*E. concinnum*	*E. menziesii*		
		ssp. *eurekense*	ssp. *menziesii*	ssp. *yadonii*
Habitat	coastal headlands	foredunes	foredunes	foredunes
Flowering season	winter, spring	winter, spring	winter, spring	summer
Life span	biennial	biennial	biennial	often perennial
Caudex	unbranched	unbranched	unbranched	branched
Leaf	subentire to toothed	toothed	lobed or irregularly toothed	subentire to lobed
Leaf succulence	usually fleshy	not obviously fleshy	fleshy	fleshy
Flower color	cream-colored to yellow	light yellow	rich yellow	rich yellow
Fruit orientation	usually erect	spreading	spreading	spreading
Fruiting pedicel	usually > 10 mm	(5-) 9 – 15 mm	3 – 9 (-13) mm	3 – 9 mm
Fruit length	3 – 13 cm	(5-) 8 – 14 cm	3 – 8 cm	3 – 8 cm
Fruit width	2 – 5 mm	2 – 3 mm	2 – 4 mm	2 – 4 mm

Determining the affinities of the Lake Earl population has important ramifications for its legal protection under the federal and California Endangered Species Acts. *Erysimum concinnum* is rare but not listed as an endangered species. *Erysimum menziesii* is listed as endangered under both the federal and California Endangered Species Acts.

Isozyme traits are inherited separately from morphologic traits and therefore provide an independent line of evidence for assessing the relatedness of populations. Isozyme analysis was chosen for examining the affinity of the Lake Earl population to *E. concinnum* and *E. menziesii* populations.

METHODS

Sample Preparation. During early 2003, leaf samples were collected from *Erysimum* plants in five populations along the northern California coast (Table 2) and shipped to NFGEL on ice. For each individual, leaf tissue was prepared by submerging an approximately 1 cm long section of leaf (40 mm^2) in 100 µl of Melody/Neale extraction buffer (USDA Forest Service 2003). Plates were stored at -70°C. On the morning of electrophoresis, the samples were thawed, ground, and soaked onto 3mm wide wicks made of Whatman 3MM chromatography paper.

Table 2. Collection locations of wallflower (*Erysimum*) samples used in this study. All locations are in north coastal California.

Taxon	Location	City	County
E. concinnum	Whaler Rock	Crescent City	Del Norte
Lake Earl Dunes	Lake Earl Dunes	Crescent City	Del Norte
E. menziesii ssp. eurekense	Lanphere Dunes, Humboldt Bay	Eureka	Humboldt
E. menziesii ssp. menziesii	McKerricher State Park	Ft. Bragg	Mendocino
E. menziesii ssp. yadonii	Marina Bay State Park	Monterey	Monterey

Electrophoresis. Methods of sample preparation and electrophoresis follow the general methodology of Conkle et al. (1982), with some modifications (USDA Forest Service 2003). All enzymes were resolved on 11% starch gels. A lithium borate electrode buffer (pH 8.3) was used with a Tris citrate gel buffer (pH 8.3) (Conkle et al. 1982) to resolve fluorescent esterase (FEST), malic enzymes (ME), and phosphoglucose isomerase (PGI). A sodium borate electrode buffer (pH 8.0) was used with a Tris citrate gel buffer (pH 8.8) (Conkle et al. 1982) to resolve aspartate aminotransferase (AAT), triosephosphate isomerase (TPI), and uridine diphosphoglucose pyrophosphorylase (UGPP). A morpholine citrate electrode and gel buffer (pH 6) (USDA Forest Service 2003) was used to resolve esterase (EST), malate dehydrogenase (MDH), and phosphogluconate dehydrogenase (6PGD). Enzyme stain recipes follow USDA Forest Service (2003). Two loci were resolved for EST, PGI, and UGPP, and three were resolved for FEST, for a total of 14 loci. All 14 loci consistently resolved in all samples.

For quality control, 10% of the individuals were run and scored twice. Gels were photographed, and the photographs consulted to resolve quality control issues. Genetic interpretations were inferred directly from isozyme phenotypes based on knowledge of the generally conserved enzyme substructure, compartmentalization, and isozyme number in higher plants (Gottlieb 1981; 1982; Weeden and Wendel 1989).

Data Analysis. Isozymes were scored as if the plants were diploid, although these plants may be polyploid. Like other members of the *Erysimum capitatum* complex, *E. menziesii* is reported to have n = 18 (Price 1993), and some other North American *Erysimum* species have n = 6, 7, or 8 (Price 1993, Missouri Botanic Garden 2004). PGI appeared to be tetraploid and was partitioned into two loci. Certain other loci that exhibited fixed heterozygosity were treated as diploid and heterozygous. Therefore, observed heterozygosity is not a reliable statistic and it is not reported.

Diversity statistics were calculated using Popgene (Yeh 1997). Assessment of population similarities began by converting diploid genotypes to allelic scores by Smouse and Williams (1982), which reflect the frequency of each allele for a diploid genotype. These data were analyzed by canonical discriminate analysis in two ways: the first was with all populations, including the unknown. In this analysis, the unknown is regarded as a valid taxon and we test the hypothesis that the unknown significantly differs from the other taxa. In the second analysis, the unknown is treated as an unclassified taxon, and in the analysis, individual genotypes in the unknown are classified with respect to the other taxa. These analyses were done both in SAS (v6.12) (SAS Institute, Inc. 1989), and JMP (v5) (SAS Institute, Inc. 2003). Graphical presentation of data in the canonical analysis was in JMP.

RESULTS

Genetic Interpretation. Most enzymes were easily scored under the assumption that they were diploid. One enzyme, PGI, had the complicated, variable band patterns of a tetraploid with tetrasomic inheritance. For analysis, it was scored as two loci and variation was partitioned evenly between them. That is, a score of 1122 was divided into scores of 12 for each locus, not 11 and 22. To investigate the effect of the genetic interpretation of this enzyme system on the final results, PGI was also scored as two loci with variation assigned to the second locus (data not shown). Here a score of 1122 was divided into scores of 11 and 22. Final results remained the same with either interpretation of PGI. Therefore, the scores that partitioned the variation between the two loci were included in the final dataset. Three additional loci showed the fixed heterozygosity of tetraploids with disomic inheritance. These were AAT in *E. concinnum* and the Lake Earl Dune population, UGPP2 in *E. menziesii* ssp. *eurekense* and *E. menziesii* ssp. *yadonii,* and PGI2 in *E. menziesii* ssp. *eurekense* (Appendix A). These enzymes were treated as diploid and heterozygous. All the other enzymes had the alternate genotypes (e.g. homozygous for allele 1, heterozygous, and homozygous for allele 2) typical of diploids or of polyploids with tetrasomic inheritance. They were scored as diploid.

Genetic Diversity. Except for the *Erysimum menziesii* ssp. *eurekense* population, which had no variation within the population at all (each individual had the same isozyme genotype), the wallflower populations were highly variable (Table 3), with 35% to 57% polymorphic loci. The Lake Earl Dunes population was among the more variable. These wallflowers obviously reproduce sexually; except in the invariant *E. menziesii* ssp. *eurekense,* most 14-locus genotypes were unique and the maximum number of individuals sharing a genotype was three (data not shown).

Table 3. Genetic variation in wallflowers (*Erysimum*) of north coastal California, based on 14 isozyme loci treated as diploid. N = sample size. P = percent polymorphic loci. A = alleles per locus. Ae = effective alleles per locus (a measure of the evenness of allele frequencies). I = Shannon information index (a measure of variability). s.e. = standard error. s.d. = standard deviation. The *E. menziesii* ssp. *eurekense* population had no variation (0% polymorphic loci, 1 allele per locus); the variation reported in the table results from fixed heterozygosity.

Taxon	N	P	A	(s.e.)	Ae	(s.d.)	I	(s.d.)
Lake Earl Dunes	24	57	1.8	(0.2)	1.3	(0.4)	0.318	(0.32)
E. concinnum	25	43	1.8	(0.3)	1.4	(0.7)	0.331	(0.45)
E. menziesii, total	75	57	2.4	(1.9)	1.7	(0.9)	0.488	(0.53)
ssp. *eurekense*	25	14	1.1	(0.1)	1.1	(0.4)	0.099	(0.25)
ssp. *menziesii*	25	35	1.6	(0.3)	1.4	(0.9)	0.280	(0.47)
ssp. *yadonii*	25	50	1.9	(0.3)	1.4	(0.5)	0.367	(0.43)
All wallflowers examined	129	71	2.8	(1.9)	1.7	(0.7)	0.568	(0.48)

Taxonomy. Few fixed differences distinguish populations (Appendix B). All individuals of *Erysimum menziesii* ssp. *menziesii* have allele 2 for FEST4. All individuals of *E. concinnum* have allele 3 for FEST1. If *E. concinnum* and the unknown Lake Earl Dunes population are considered conspecific, that species is separated from *E. menziesii* by AAT allele 2, for which they are presumably homozygous in at a second AAT locus. All individuals of *E. menziesii* ssp. *eurekense* had allele 6 for PGI, an allele not seen in any other population.

The populations were very highly differentiated, and variation among individuals (F_{IS} = 10.1%) made a much smaller contribution to the total variation than did variation among populations (F_{ST} = 46.4%). This was true even when the analysis was run without the invariant *E. menziesii* ssp. *eurekense* population (F_{IS} = 1.8%, F_{ST} = 39.7%). The high degree of population differentiation may be related to the geographic isolation of these populations, or it may reflect their taxonomic distinctions; only one population per taxon was sampled. Genetic similarities among the populations ranged from 0.72 to 0.89. The two most similar populations were *E. concinnum* and the unknown from Lake Earl Dunes; the two least similar populations were *E. concinnum* and *E. menziesii* ssp. *eurekense*.

Discriminate analysis produced three clusters of wallflower populations: *Erysimum concinnum* + unknown; *E. menziesii* ssp. *menziesii* + *E. menziesii* ssp. *yadonii;* and the invariant *E. menziesii* ssp. *eurekense* population (Figure 1). Only seven (5.6%) of the individuals might be considered misclassified, in that they were much more similar to another population than to the one in which they originated (Table 5). Five of these were *E. concinnum* individuals that were more similar to the unknown Lake Earl Dunes population than to other *E. concinnum*. Interestingly, one individual from the Lake Earl Dunes population was more similar to *E. menziesii* ssp. *menziesii* than to other members of its own population.

Table 5. Discriminate scores for possibly misclassified individuals from Discriminate Analysis Canonical Plot based on wallflower (*Erysimum*) isozymes. Predicted = the population that the program calculated the individual belongs to. Prob(Actual) is the probability that the individual belongs to the population from which it came. Prob(pred) is the probability that the individual belongs to the population the analysis predicts it belongs to.

Actual identity	Dist (Actual)	Prob (Actual)	-Log (Prob)	Plot –Log (Prob)	Predicted	Prob (Pred)
E. concinnum	-62.1640	0.0304	3.492		Lake Earl Dunes	0.9696
E. concinnum	-65.5791	0.2780	1.280		Lake Earl Dunes	0.7220
E. concinnum	-61.5952	0.1134	2.177		Lake Earl Dunes	0.8866
E. concinnum	-65.5239	0.0863	2.450		Lake Earl Dunes	0.9137
E. concinnum	-54.5729	0.4967	0.700		Lake Earl Dunes	0.5033
E. m ssp. *yadonii*	-44.5944	0.0000	10.229		*E. m. ssp. menziesii*	1.0000
Lake Earl Dunes	-42.1741	0.1474	1.915		*E. m. ssp. menziesii*	0.8525

DISCUSSION

The Lake Earl Dunes population clearly clusters with the *Erysimum concinnum* population, not the *E. menziesii* populations (Figure 1). Isozyme analysis suggests that it may be conspecific with *E. concinnum.* Conspecific populations usually have genetic similarities of 0.9 or higher (Crawford 1989); the similarity of the Lake Earl Dunes population and *E. concinnum* is 0.89, greater than similarities to the other taxa (which range from 0.72 to 0.81). The Lake Earl Dunes population is very distinct from the *E. menziesii* ssp. *eurekense* population.

Some evidence hints at an affinity between the Lake Earl Dunes population and *Erysimum menziesii,* rather than *E. concinnum.* For one thing, the isozyme profile of an individual from the Lake Earl Dunes population is much more similar to *E. menziesii* than to *E. concinnum* (Figure 1; Table 5). Also, all individuals of *E. concinnum* are homozygous for FEST1 allele 3; this allele is not seen in any individual from the Lake Earl Dunes population, or in any tested *E. menziesii* individual. Such fixed differences usually distinguish species, not nearby

populations of a single species. These *Erysimum* occurrences are small and isolated so a fixed difference might become established in the population by genetic drift. Also, the Lake Earl Dunes population is similar to *E. menziesii* ssp. *eurekense* in height, pubescence, flower color, and habitat.

Taxonomic decisions should be made based on the totality of available evidence. Isozymes are useful taxonomically because they provide data independent of morphology and because they are much less likely to be affected by the environment than are morphological traits, but they do not cancel out morphology. All traits should be considered when deciding whether to classify the Lake Earl Dunes wallflower population as *Erysimum concinnum* or *E. menziesii.*

There are several ways to treat taxonomically the contradictory set of data observed in the Lake Earl Dunes population:

(1) Classify the Lake Earl Dunes population as *E. concinnum* based on isozymes, and treat fruit orientation, the single morphological trail uniting the Lake Earl Dunes population with *E. concinnum* as more taxonomically important (because it is correlated with more genetic differentiation) than had been realized. The Lake Earl Dunes population would be anomalous within *E. concinnum* in habitat and several morphological traits. It would lack the special protection given endangered species.

(2) Consider the Lake Earl Dunes population intermediate. Treat it as a population of hybrid origin or exhibiting introgression.

(a) Make no taxonomic changes. *E. concinnum* remains a rare but unlisted species with one more known population, and the status of *E. menziesii* is unaffected.

(b) Classify it as *Erysimum menziesii* based on several morphological traits, but hold *E. concinnum* as distinct on the basis of the differences in morphology and habitat and the fixed difference in FEST1 alleles. This would give the Lake Earl Dunes population greater administrative protection, but would be difficult to reconcile with the close similarity between isozymes of the Lake Earl Dunes population and *E. concinnum.*

(3) Treat the unique combination of characters found in the Lake Earl population as a third species. This might be hard to defend given the relatively high genetic similarity (0.89) of the Lake Earl population to *E. concinnum* and its morphological similarity to *E. menziesii* spp. *eurekense,* but this is a way to deal with the contradictory information about this population's affinities.

The human need to fit plants into neat, mutually exclusive species categories often, but not always, coincides with neat discontinuities with its biological variation (Hey 2001). Unfortunately, these coastal *Erysimum* populations are among the exceptions. These wallflowers are highly variable. They form metapopulations that are genetically isolated because they are geographically isolated. These metapopulations are differentiated from one another morphologically, but the distinctions are small. The complex is probably actively speciating. Virtually any taxonomic treatment, from classifying them as a single, extraordinarily variable species to recognizing each metapopulation as a distinct species would convey some useful biological information but obscure other important data.

FURTHER RESEARCH

This study involved just one population per taxon. The taxonomic position of the Lake Earl Dunes population might be better understood if more were known about variation within each taxon. Specifically, does allele 3 at the FEST1 locus (which distinguishes the *E. concinnum* population sampled from the Lake Earl Dunes population) characterize all *E. concinnum* populations, or does this trait vary within the species? The complete lack of isozyme variation in the *E. menziesii* ssp. *eurekense* population sampled may result in an incomplete understanding of the relationship between the Lake Earl Dunes population and *E. menziesii* ssp. *eurekense.* Other *E. menziesii* ssp. *eurekense* populations exist and could be sampled. A common garden study in the greenhouse or in a field site distant from extant *Erysimum* populations (to prevent gene exchange with extant populations) might clarify morphological similarities among the taxa.

LITERATURE CITED

Conkle, M. T., Hodgskiss, P. D., Nunnally, L. B., and Hunter, S. C. 1982. Starch Gel Electrophoresis of Conifer Seeds: a Laboratory Manual. Gen. Tech. Rep. PSW-64. Berkeley, CA: Pacific Southwest Forest and Range Experiment Station, Forest Service, U.S. Department of Agriculture. 18pp.

Crawford, D. J. 1989. Enzyme electrophoresis and plant systematics. Pp. 146-164 *in* D. E. Soltis and P. S. Soltis, eds., Isozymes in Plant Biology. Dioscorides Press, Portland, Oregon.

Gottlieb, L. D. 1981. Electrophoretic evidence and plant populations. Prog. Phytochem. 7: 1-46.

Gottlieb, L. D. 1982. Conservation and duplication of isozymes in plants. Science 216: 373-380.

Hey, J. 2001. Genes, Categories, and Species; the Evolutionary and Cognitive Causes of the Species Problem. Oxford University Press, New York. 217 pp.

Missouri Botanic Garden. accessed 2004. Index to Plant Chromosome Numbers (IPCN). http://mobot.mobot.org/W3T/Search/ipcn.html

Price, R. A. 1987. Systematics of the *Erysimum capitatum* Alliance (Brassicaceae) in North America. Ph.D. Thesis. University of California, Berkeley. 208 pp.

Price, R. A. 1993. *Erysimum.* pp. 421 – 422 *in* J. C. Hickman, ed., The Jepson Manual; Higher Plants of California. University of California Press, Berkeley, California.

SAS Institute Inc. 1989. SAS/STAT™ User's Guide, Version 6, Fourth Edition, Volume 1. SAS Institute Inc., Cary, NC.

SAS Institute Inc. 2003. JMP® Statistics and Graphics Guide. SAS Institute Inc., Cary, NC.

Smouse, P. E., and Williams, R.C. 1982. Multivariate analysis of HLA-disease associations. Biometrics 38:757-768.

USDA Forest Service. 2003. National Forest Genetic Electrophoresis Laboratory Standard Operating Procedures. NFGEL, USDA Forest Service, Placerville, California.

Weeden, N. F., and Wendel, J. F. 1989. Genetics of plant isozymes. Pp. 46-72 *in* D. E. Soltis and P. S. Soltis, eds. Isozymes in Plant Biology, Dioscorides Press, Portland, Oregon.

Yeh, F. C., Yang, R.-C., and Boyle, T. 1997. Popgene Version 1.32. University of Alberta, Edmonton, Alberta.

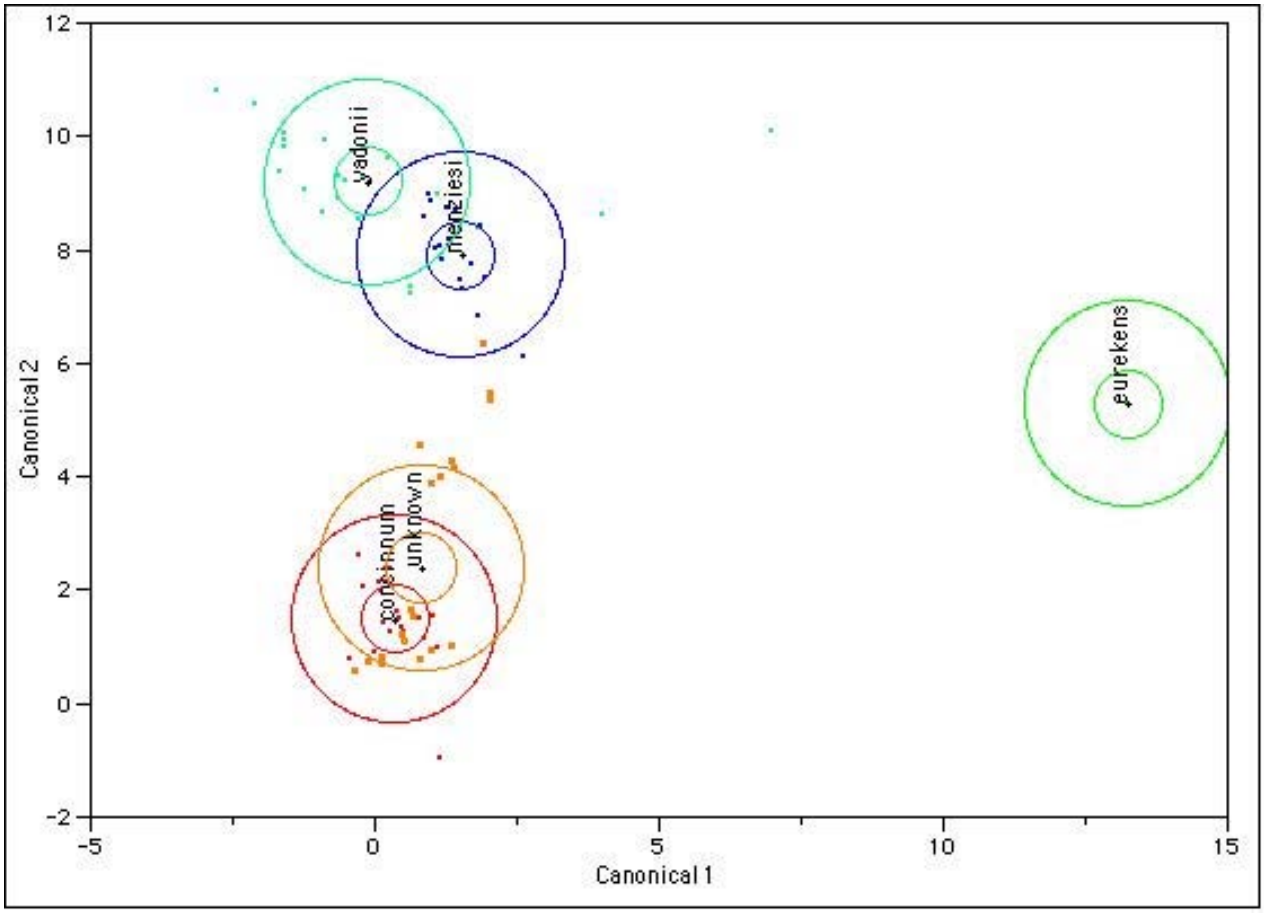

Figure 1. Discriminate Analysis Canonical Plot based on wallflower (*Erysimum*) isozymes.

APPENDIX A. Genotypes at each locus in each wallflower (*Erysimum*) population sampled from north coastal California. Numbers in **boldface** type indicate fixed heterozygosity; all individuals have what seems to be a heterozygous genotype because actually they have two copies of the gene, with a different allele in each copy. "PGI tetra" = the tetraploid genotype that was partitioned to produce the two PGI 'loci' shown.

Species	ssp.	FEST1	FEST2	FEST4	PGI1	PGI2	AAT	UGPP1	UGPP2	EST1	EST2	MDH3	6PGD1	ME	TPI	PGI tetra
E. concinnum		33	11 13 33	33	11 13 15 17 55	11 13 15 17 35 55 57	**12**	12 22	33	11	11 12 22 33	11	11	11	11	1111 1113 1115 1117 1133 1135 1155 1157 1177 5555
E. unknown		11 12 22	33 34 44	33	11 15 17	11 15 17	**12**	11 12 22	33	11 22	11 12 33	11	11	11	11	1111 1115 1117 1155 1177
E. menziesii	*eurekense*	11	33	11	11	**16**	11	22	**34**	11	11	11	11	11	11	1116
E. menziesii	*menziesii*	11	11 13 33	22	12 15 17 22 25 27	12 15 17 22 25 27 55 57 77	11	22	33	11 12 22	12 22	11	11	11	11	1122 1155 1177 1777 2222 2225 2277 2555 2557
E. menziesii	*yadonii*	11	11 12 13	11 13 33	11 12 13 14 23	11 12 13 14 23 44	11	11 15 22 25 55	**34**	11	11 12 22	11	11	11	11	1111 1114 1122 1133 1144 1444 2233

APPENDIX B. Isozyme allele frequencies in wallflower (*Erysimum*) populations located in north coastal California. Four additional loci (MDH3, ME, TPI, and 6PDG1) showed no variation.

Locus	allele	*E. concinnum*	Lake Earl	*E. m. eurekense*	*E. m. menziesii*	*E. m. yadonii*
AAT	1	0.5000	0.5000	1.0000	1.0000	1.0000
AAT	2	0.5000	0.5000			
EST1	**1**	**1.0000**	**0.9583**	**1.0000**	**0.8000**	**1.0000**
EST1	**2**		**0.0417**		**0.2000**	
EST2	1	0.2000	0.6875	1.0000	0.0800	0.6800
EST2	2	0.6800	0.2708		0.9200	0.3200
EST2	3	0.1200	0.0417			
FEST1	**1**		**0.2500**	**1.0000**	**1.0000**	**1.0000**
FEST1	**2**		**0.7500**			
FEST1	**3**	**1.0000**				
FEST2	1	0.3800			0.2600	0.7800
FEST2	2					0.1800
FEST2	3	0.6200	0.8958	1.0000	0.7400	0.0400
FEST2	4		0.1042			
FEST4	**1**			**1.0000**		**0.0600**
FEST4	**2**				**1.0000**	
FEST4	**3**	**1.0000**	**1.0000**			**0.9400**
PGI1	1	0.6800	0.8125	1.0000	0.2000	0.6800
PGI1	2				0.5200	0.2000
PGI1	3	0.0600				0.0600
PGI1	4					0.0600
PGI1	5	0.1400	0.0417		0.1800	
PGI1	6					
PGI1	7	0.1200	0.1458		0.1000	
PGI2	**1**	**0.4600**	**0.7083**	**0.5000**	**0.1800**	**0.6000**
PGI2	**2**				**0.3200**	**0.2000**
PGI2	**3**	**0.1000**				**0.0600**
PGI2	**4**					**0.1400**
PGI2	**5**	**0.2400**	**0.0625**		**0.2800**	
PGI2	**6**			**0.5000**		
PGI2	**7**	**0.2000**	**0.2292**		**0.2200**	
UGPP1	1	0.0600	0.2917			0.1400
UGPP1	2	0.9400	0.7083	1.0000	1.0000	0.3000
UGPP1	5					0.5600
UGPP2	**3**	**1.0000**	**1.0000**	**0.5000**	**1.0000**	**0.5000**
UGPP2	**4**			**0.5000**		**0.5000**

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Final Report

Isozyme Analysis of *Arnica lessingii*

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SUMMARY

1. **Should *Arnica lessingii* ssp. *norbergii* and *A. lessingii* ssp. *lessingii* be considered subspecies?**
   Isozyme analysis shows that these taxa are closely related. In itself, their close genetic relationship tells us nothing about their appropriate taxonomic status; subspecies usually are closely related. To answer the taxonomic question, we need to compare genetic variation in *A. lessingii* ssp. *norbergii* to the variation in *A. lessingii* ssp. *lessingii*, but the *A. lessingii* ssp. *lessingii* sample in this study consists of only six individuals of one population. Of the total number of plants sampled, 83% contained the same multilocus genotype, and very little variation occurred within the study. Therefore, no pattern was detected that shed light on the appropriate taxonomic determination.
2. **How genetically variable is the rare *A. lessingii* ssp. *norbergii?***
   A. lessingii ssp. *norbergii* has little genetic variation detectable by isozyme electrophoresis. A single multilocus genotype was the only or the most common genotype in all four populations sampled. Only two genes (UGPP and IDH) showed any variation; they had two alleles each.
3. **How genetically distinct are the four *A. lessingii* ssp. *norbergii* populations sampled?**
   Genetic similarity was high among *A. lessingii* ssp. *norbergii* populations because most genes had no variation. However, variation was patterned geographically. One rare allele was confined to two populations (from Harlequin Lake and Chichagof Island). The other rare allele was found in three of the four populations (Portage Area, Harlequin Lake, and Chichagof Island). The differences among populations may result from founder effects or genetic drift in small populations. The most distinct *A. lessingii* ssp. *norbergii* populations were the Harlequin Lake and Harlequin Trail populations, located only about 1.2 miles apart.
4. **What is the significance of the fact that isozymes are identical in the Harlequin Trail population of *A. lessingii* ssp. *norbergii* and the one *A. lessingii* ssp. *lessingii* population sampled?**
   The significance of these two populations sharing the same common genotype may be minimal. (1) The sample from the Baranof population of *A. lessingii* ssp. *lessingii* consists of only six individuals. (2) Sampling only one population of this widespread taxon does not show the pattern of variation in *A. lessingii* ssp. *lessingii*. (3) Most important, the common, widespread alleles shared by these two populations are probably shared because they were inherited from a recent common ancestor. Whether *A. lessingii* ssp. *norbergii* and *A. lessingii* ssp. *lessingii* should be considered subspecies or not, their morphological similarities show that they had a recent common ancestor. Therefore, this aspect of the isozyme data does not increase our knowledge of their history, gene flow, or taxonomy.
5. **Are isozymes a good tool for determining the taxonomic status of *A. lessingii* subspecies?**
   Isozymes are useful for comparing phylogenetic history or gene flow among populations *if* sufficient variation can be detected. If genetic variation in common *A. lessingii* ssp. *lessingii* is as limited as that observed in rare *A. lessingii* ssp. *norbergii*, isozymes may have little value for answering this question. However, the amount of isozyme variation in *A. lessingii* ssp. *lessingii* is unknown at this time (our sample size for this subspecies was six individuals from one population), and sometimes common taxa are more variable than related rare ones. Perhaps isozymes are good tools for addressing the taxonomic questions asked, but other tools should be investigated.

INTRODUCTION

Arnica lessingii Greene ssp. *norbergii* Hulten & Maguire (Maguire 1942) is designated as a sensitive species in the Alaska Region of the Forest Service (USDA Forest Service, Alaska Region, 2002). Plants meeting the description of *A. lessingii* ssp. *norbergii* are known from only about four locations in southern Alaska, all of them isolated from each other, though some are located near *A. lessingii* ssp. *lessingii* populations. Botanists disagree about the taxonomic status the *A. lessingii* subspecies.

Arnica lessingii Greene is widespread in alpine and subalpine meadows and in well-drained lowland meadows and open areas; with an amphi-Beringian distribution (Hulten 1968, Cody 1996). It is distinguished from other Arnicas by its purple-black anthers (yellow in most other species), and is unusual in the genus because the flower heads are nodding. Typical *A. lessingii* ssp. *lessingii* plants are easily distinguished from the strikingly taller *A. lessingii* ssp. *norbergii* (Table A).

Table A. Morphological traits distinguishing *Arnica lessingii* ssp. *lessingii* and *A. lessingii* ssp. *norbergii*.

Trait	*A. lessingii* ssp. *lessingii*	*A. lessingii* ssp. *norbergii*
Habitat	grassy ridges, tundra	shrubby open areas
Plant height	short	tall
Leaves in rosette?	usually yes; occasionally no	no
Number of stem leaves	1 – 3 (-4)	(3-) 4 – 6
Pubescence on involucre & upper stem	dense	sparse

Plants from what appear to be *Arnica lessingii* ssp. *lessingii* populations may be relatively leggy, with more stem leaves than is typical, and long-stemmed, tall plants from what appear to be *A. lessingii* ssp. *norbergii* populations may have as few as three pairs of stem leaves. The differences in stem height, leaf spacing, and even pubescence could all result from the habitat differences altering the phenotype or creating strong selection pressure to which the genotype could quickly respond. Therefore, it is understandable that botanists differ in their interpretation of *A. lessingii* variation (Table B).

Table B. Taxonomic treatment of *Arnica lessingii* ssp. *norbergii* by various botanists.

Authors	Year	Coverage	*A. lessingii* ssp. *norbergii* recognized?
Maguire	1942	Alaska and Yukon	yes; described here
Camp & Gilly	1943	broad	mentioned
Hulten	1941-1950	Alaska and Yukon; Vol. X	yes; expressed doubts on status
Anderson	1959	Alaska, adj. Canada	yes (?)
Wiggins & Thomas	1962	Alaskan arctic slope	no (not present)
Hulten	1968	Alaska, adj. Canada	yes
Welsh	1974	Alaska, adj. Canada	no; included in typical *A. lessingii*
Cody	1996	Yukon	no (not present)
Douglas et al.	1998	British Columbia	no; included in typical *A. lessingii*

Even botanists who recognize *Arnica lessingii* ssp. *norbergii* express doubts about its status. Hulten (1949) wrote: “It may be left undecided whether subsp. *norbergii* is merely a southern condition of this species . . . . The enumerated material of that plant includes several transitional types, the most pronounced variation being represented by the type specimen . . . . It differs,

however, very strikingly from the bulk of the material." Anderson (1959) wrote that ssp. *norbergii* is "perhaps only a local variation" of *A. lessingii.*

When morphological data are ambiguous as to the taxonomic status of a subspecies, genetic markers may provide insight and allow resolution of the taxonomic issue. In order for the genetic marker to be informative, however, sufficient variation must exist within the species so that any variation among subspecies may be detected. The combination of morphological and genetic data is credited with resolving taxonomic issues in a number of species (Donoghue and Sanderson 1992).

This genetic study was undertaken to assess genetic similarity between *A. lessingii* ssp. *norbergii* and the more common *A. lessingii* ssp. *lessingii*, and thus to provide evidence for evaluating their taxonomic status. Specifically, neutral bi-parentally inherited markers (isozymes) were used to characterize the genetic similarity of the two subspecies, identify any genetic structure within *A. lessingii* ssp. *norbergii*, and provide further data for the continuing debate over the taxonomic status of these two groups. Although not part of the original study objectives, a morphological assessment of the two groups was made based on provided voucher specimens and included in the report.

METHODS

Sampling. Four *Arnica lessingii* ssp. *norbergii* populations and one *A. lessingii* ssp. *lessingii* population were sampled for this study (Table 1). A total of 100 plants were collected for analysis; 94 plants from ssp. *norbergii* and 6 plants from ssp. *lessingii*. Each sample consisted of several leaves per plant. Tissue was shipped to NFGEL on ice.

Isozyme Preparation. For each individual, isozyme samples were prepared by submerging approximately 75mg of tissue in 100 µl of Gottlieb extraction buffer (USDA Forest Service 2003). Plates were stored at -70°C. On the morning of electrophoresis, the samples were thawed, ground, and soaked onto 3mm wide wicks made of Whatman 3MM chromatography paper.

Electrophoresis. Methods of sample preparation and electrophoresis follow the general methodology of Conkle et al. (1982), with some modifications (USDA Forest Service 2003). All enzymes were resolved on 11% starch gels. A lithium borate electrode buffer (pH 8.3) was used with a Tris citrate gel buffer (pH 8.3) to resolve FEST, ME, PGM, MDH, LAP, ACO, and PGI. A sodium borate electrode buffer (pH 8.0) was used with a Tris citrate gel buffer (pH 8.8) to resolve AAT, TPI, CAT, G6PD, GLYDH, PGM, and UGPP. Morpholine citrate electrode and gel buffers (pH 6 and pH 8) were each used to resolve EST, MDH, 6PGD, IDH, DIA, SKD, and MNR. Enzyme stain recipes follow USDA Forest Service (2003). MC6 and MC8 buffer systems resolved loci equally well. Only 12 loci (10 enzyme systems) resolved well enough to score (LB: FEST-1, FEST-2, ME, MDH, PGI-1, PGI-2; SB: TPI, UGPP; MC6 or 8: IDH, DIA, 6PGD, MNR).

For quality control, 10% of the individuals were run and scored twice. Gels were photographed, and the photographs consulted to resolve quality control issues. Genetic interpretations were inferred directly from isozyme phenotypes based on knowledge of the generally conserved enzyme substructure, compartmentalization, and isozyme number in higher plants (Gottlieb 1981; 1982; Weeden and Wendel 1989).

DNA Extraction. DNA extraction was carried out on leaf tissue from a total of four samples using a Qiagen DNEasy Mini Kit following the manufacturer's instructions. DNA quality and approximate quantity were assessed by visualizing all samples against an uncut Lambda DNA

standard on 0.8% agarose gels stained with EtBr under UV light. DNA was ethanol precipitated, dried down, and shipped to Dr. Katarina Andreasen, Evolutionary Biology Centre, Uppsala, Sweden, in April 2004 for analysis. Each sample is estimated to contain between 1.5-1.8 ug of high molecular weight DNA. Samples sent include: #4260 - *A. lessingii* ssp. *norbergii*, Harlequin Trail; #4306 - *A. lessingii* ssp. *norbergii*, Harlequin Lake; #4462 - *A. lessingii* ssp. *lessingii*, Baranof Island; #4566 - *A. lessingii* ssp. *norbergii*, Portage Area. All four samples have the same common isozyme multilocus genotype (see Results).

Isozyme Data Analysis. Isozymes were scored as if the plants were diploid, although many species in the genus *Arnica* are polyploid. Banding patterns were very simple, with most loci appearing as a single band. Diversity statistics were calculated using Biosys (Swofford and Selander 1989).

RESULTS

Little isozyme variation was found in *Arnica lessingii* (Table 2). Of the 12 enzymes tested, 10 were monomorphic. The variations were a null allele in UGPP and an active rare allele in IDH. Seventy-nine individuals sampled in the study were monomorphic for the same multilocus genotype (the 'common genotype'). This common genotype occurred in each sampled plant from the Baranof Island population of ssp. *lessingii*, as well as the Harlequin Trail population of ssp. *norbergii*. The three other ssp. *norbergii* populations sampled were predominately comprised of plants with the common genotype (Harlequin Lake = 63%, Portage Area = 70%, and Chichagof Island = 75%). Of the remaining 21 samples genotyped in the study, 13 contained variation in the UGPP locus, three individuals contained variation in the IDH locus, and five plants contained variation in both loci.

Genetic similarities among *A. lessingii* populations were greater than 0.99 (Table 3), high even for conspecific plants. However, this great similarity resulted from the lack of variation at most loci. All populations were identical at 83% of the loci examined, and the widespread multilocus genotype was the most common even in the populations that had some variation.

What little genetic variation could be detected was structured by population (Table 4), and therefore the population-level variation within total variation (Fst value) of 0.14 indicates that the population differentiation was moderately great (Table 5). A rare allele for IDH was found in only two populations, Harlequin Lake and Chichagof Island. A null allele for UGPP was found in the *A. lessingii* ssp. *norbergii* populations Harlequin Trail, Portage Area, and Chichagof Island. All six small subpopulations of the Portage area population contained both the common and null alleles for UGPP (Table 1). Although isozyme variation in *A. lessingii* var. *norbergii* was patterned geographically, there was more variation within populations than among them (Table 5).

DISCUSSION

Morphology. Sometimes identification keys hinder understanding plant taxa as much as they help. That may be the case with the *Arnica lessingii* subspecies. When a key offers a choice that is a point on a continuum, like "Stem leaves in 2-3 pairs" vs. "Stem leaves in 4-6 pairs" separating the two subspecies (Hulten 1968), some plants are likely to transgress this arbitrary boundary. For example, two collections from the Harlequin Trail population sampled in this study (*Trull 304* and *Trull 305;* TNFS Herbarium) key to different subspecies although the plants look very much alike.

Botanists who recognize both subspecies seem to be separating two growth forms of *Arnica lessingii*. In one (*A. lessingii* ssp. *lessingii*), the stem elongates very little during its early growth, so the internodes are short, the leaves are close together, forming a rosette, and few leaves are produced (Table A). In the other (*A. lessingii* ssp. *norbergii*), the stem elongates more, internodes are longer, leaves are further apart, no rosette is formed, and more leaves are usually produced. Of course, intrapopulation variation may occur, and indeed some *A. lessingii* ssp. *lessingii* specimens include two or three typical, rosette-forming plants and one leggier plant with no rosette and maybe three pairs of stem leaves.

Applying these criteria to the two specimens from the supposedly mixed Harlequin Trail population, we see that both specimens (*Trull 304* and *Trull* 305) have the same growth form. Both have internodes relatively elongated throughout the stem, indicating that stem elongation was more uniform. Both meet the criteria for *Arnica lessingii* ssp. *norbergii,* although some of the plants (segregated as *Trull 305*) have only three pairs of stem leaves. This population is not mixed.

Other samples, however, appear transitional between the two subspecies. For example, *Williams 11390* (at OSC), from Mt. Roberts, Juneau, consists of plants of intermediate height and lacking a rosette, but with three separated pairs of stem leaves. These plants can be keyed to be *Arnica lessingii* ssp. *lessingii,* but are not typical. Plants like these caused Hulten (1941-1950) to question whether *A. lessingii* ssp. *norbergii* might be "a southern condition of this species" rather than a taxonomic unit best treated as a subspecies.

Genetic Diversity and Taxonomy. The existence of populations with intermediate morphology is not the only reason to question the usefulness of treating the leggy southern *Arnica lessingii* populations taxonomically as a subspecies. The differences in stem elongation and number of leaves, and perhaps even the difference in pubescence, could result from a very limited genetic base. Assuming that these differences are genetic, they could appear quickly, results of strong selection pressure on few genes. Greater genetic differentiation is not a requirement for subspecies status – there are no clear standards for subspecies status – but if the *A. lessingii* ssp. *lessingii* and *A. lessingii* ssp. *norbergii* populations differed in more traits (implying great genetic differentiation), the case for treating the two groups of populations taxonomically would be stronger. Morphological, isozyme, or DNA differences might all be useful to strengthening the case for treating the two as subspecies.

Unfortunately, the limited sampling of *Arnica lessingii* ssp. *lessingii* for this study prevents any detailed comparison of the two subspecies. The only genotype detected in the six *A. lessingii* ssp. *lessingii* samples is the same genotype that is most common in all the *A. lessingii* ssp. *norbergii* populations sampled, but this could simply indicate that the populations all have a recent common ancestor. This is already apparent from their morphological similarity and is expressed by their taxonomic status as subspecies. Genetic similarity of different subspecies is usually high, comparable to that of populations of the same subspecies (Crawford 1989).

The presence of both the common and null alleles for UGPP in all six small subpopulations of the Portage area suggests that gene flow occurs among these subpopulations, all of which are located close together on one trail. However, although the genetic variation detected was geographically patterned in ssp. *norbergii*, that variation may result from founder effects or genetic drift, rather than historic patterns of species ranges or gene flow. Evidence for this is provided by the Harlequin Lake and Harlequin Trail populations, which were only about 1.2 miles apart but had the lowest genetic similarity of 0.991 among *A. lessingii* ssp. *norbergii* populations.

Often isozymes clarify taxonomic puzzles like that presented by *Arnica lessingii* ssp. *norbergii*. However, isozymes are useful for comparing phylogenetic history or gene flow among populations only when sufficient variation can be detected. If genetic variation in common *A. lessingii* ssp. *lessingii* is as limited as that observed in rare *A. lessingii* ssp. *norbergii,* isozymes may have little value for assessing the taxonomic status of the subspecies. In that case, more variable markers like the DNA variation detected in ISSRs might be useful. Regardless of which tools are used to assess variation in this species, an element of taxonomic judgment will remain.

Ecological Variation. Since the isozyme data did not assess sufficient levels of variations to address the goal of taxonomic determination, we thought it would be beneficial to explore the larger issue of variation within *Arnica*. One possibility exists that *Arnica lessingii* ssp. *norbergii* is an ecological variant of *A. lessingii,* with its implication that the plant's status as an ecological variant precludes its being a subspecies. An ecological variant might be a phenotypic variation on a constant genetic background. *Arnica lessingii* ssp. *norbergii* lives at lower elevations with more shrub vegetation than does *Arnica lessingii* ssp. *lessingii*. That habitat difference might induce purely phenotypic differences (including stem elongation and thus more leaves on the stem, and loss of pubescence), and such differentiation would certainly not warrant taxonomic recognition. This hypothesis could be tested in a common garden, if the investment in time and resources seemed worthwhile. Additionally, gardeners who grow *Arnica lessingii* in rock gardens may have observations on the phenotypic plasticity of this species in different habitats. (Does it get "leggy" when given more water or fertilizer?)

Alternatively, an ecological variant might have differentiated repeatedly from more typical *A. lessingii* stock. Strong selection pressure in an alternate habitat (with more competition from other plants) could favor the certain genetic variants in several areas. This selection could produce gene-based physiological and morphological differences, such as the growth form distinctions between the two named subspecies. Botanists might disagree about how, or whether, to handle this differentiation taxonomically. In this case, the informal category of the ecotype might well be applied, but treating these series of populations as subspecies could also be justified.

Note that although this discussion is worded as if *Arnica lessingii* ssp. *norbergii* is the more recently evolved entity, *Arnica lessingii* ssp. *lessingii* might be a recent descendent of *Arnica lessingii* ssp. *norbergii* progenitors. All of these scenarios could be complicated and obscured by subsequent gene flow between the units we now call subspecies, and/or founder effect and genetic drift in what are now small, isolated *A. lessingii* ssp. *norbergii* populations.

What pattern of variation would help resolve this taxonomic question? If, using some adequately variable traits other than growth form and isozymes, the *Arnica lessingii* ssp. *norbergii* populations are found to be more similar to each other than to *A. lessingii* ssp. *lessingii* populations, they are probably differentiated enough to be called subspecies. If such traits suggest that *A. lessingii* ssp. *norbergii* populations are more similar to nearby *A. lessingii* ssp. *lessingii* populations than to each other, the case for calling them distinct subspecies is weakened. However, that similarity to nearby *A. lessingii* ssp. *lessingii* populations might result from recent gene flow and might be disregarded in making this taxonomic decision. Unfortunately, isozymes were not able to detect adequate levels of genetic variation capable of shedding lights on subspecific classification in *Arnica lessingii*.

LITERATURE CITED

Anderson, J. P. 1959. Flora of Alaska and Adjacent Parts of Canada. Iowa State University Press, Ames, Iowa. 543 pp.

Camp, W. H., and C. L. Gilly. 1943. Part II: Systematic Treatment [*Arnica spathulata – Arnica sachalinensis*]. Brittonia 4:457-493.

Cody, W. J. 1996. Flora of the Yukon Territory. National Research Council of Canada Press, Ottawa, Ontario. 643 pp.

Conkle, M. T., P. D. Hodgskiss, L. B. Nunnally, and S. C. Hunter. 1982. Starch Gel Electrophoresis of Conifer Seeds: a Laboratory Manual. General Technical Report PSW-64. Berkeley, California: Pacific Southwest Forest and Range Experiment Station, Forest Service, U.S. Department of Agriculture. 18pp.

Crawford, D. J. 1989. Enzyme electrophoresis and plant systematics. Pp. 146-164 *in* D. E. Soltis and P. S. Soltis, eds., Isozymes in Plant Biology. Dioscorides Press, Portland, Oregon.

Donoghue, M. J., and M. J. Sanderson. 1992. The suitability of molecular and morphological evidence in reconstructing plant phylogeny. Pages 340 - 368 *in* P. S. Soltis, D. E. Soltis, and J. J. Doyle, editors. Molecular Systematics of Plants. Chapman & Hall, New York, NY, USA.

Douglas, G. W., G. B. Straley, D. Meidinger, and J. Pojar (eds.). 1998. Illustrated Flora of British Columbia, Volume 1. British Columbia Ministry of Environment, Lands, and Parks, and Ministry of Forests.

Gottlieb, L. D. 1981. Electrophoretic evidence and plant populations. Progress in Phytochemistry 7:1-46.

Gottlieb, L. D. 1982. Conservation and duplication of isozymes in plants. Science 216:373-380.

Hulten, E. 1941-1950. Flora of Alaska and Yukon, 1-10. Lunds Universitets Arsskrift N.F., Avd. 2. vol. 37-46. Lund, Sweden.

Hulten, E. 1949. Flora of Alaska and Yukon, Volume 10. C. W. K. Gleerup. Lund, Sweden.

Hulten, E. 1968. Flora of Alaska and Neighboring Territories. Stanford University Press, Standford, California.

Maguire, B. 1942. *Arnica* in Alaska and Yukon. Madroño 6:153-155.

Swofford, D. L., and R. B. Selander. 1989. BIOSYS-1, a computer program for the analysis of allelic variation in population genetics and biochemical systematics, release 1.7 edition. Illinois Natural History Survey, Champaign, Ill.

USDA Forest Service, Alaska Region. 2002. Regional Foresters Sensitive Species List.

USDA Forest Service. 2003. National Forest Genetic Electrophoresis Laboratory Standard Operating Procedures. NFGEL, USDA Forest Service, Placerville, California.

Weeden, N. F., and J. F. Wendel. 1989. Genetic of plant isozymes. Pages 46-72 *in* D. E. Soltis and P. S. Soltis, editors. Isozymes in plant biology. Dioscorides Press, Portland, Oregon, USA.

Welsh, S. L. 1974. Anderson's Flora of Alaska and Adjacent Parts of Canada. Brigham Young University Press, Provo, Utah. 724 pp.

Wiggins, I. L., and J. H. Thomas. 1962. A Flora of the Alaskan Arctic Slope. Arctic Institute of North America Special Publication #4. University of Toronto Press. 425 pp.

Table 1. Collection sites for *Arnica lessingii.* All sites are in southeastern Alaska, except that the sites at the Portage Glacier are in south central Alaska. Collections were made in six subpopulations in the Portage area.

Taxon	N	Location	Latitude	Longitude	Elev.	Habitat	Date	Collector
ssp. *lessingii*	6	**Baranof Island**, 10 km east of Sitka on Bear Mountain	57°03'04"N	135°10'18"W	980 m	scree slope	9 Aug. 2003	Brad Krieckhaus 131
ssp. *norbergii*	30	**Harlequin Trail,** 45 km SE of Yakutat, ¾ of the way along the trail from Harlequin Lake Cabin to Harlequin Lake	59°25 00"N	139°00'39"W	30 m	open shrubby area	28 July 2003	Mary Stensvold 8154
	30	**Harlequin Lake,** about 45 km SE of Yakutat, along shore of Lake	59°24'30"N	139°00'22"W	25 m	open shrubby area	28 July 2003	Mary Stensvold 8153
	30	**Portage Area**, 100 m south of the Begich-Boggs Visitor Center, along the Moraine Trail			< 30 m	open shrubby area	19 August 2003	Brooke Steiner s.n.
	(4)	subpopulation 1: under bench at start of turnaround	60°46'58"N	148°50'37"W				
	(6)	subpopulation 2: near bottom step of stairway	60°46'58"N	148°50'36"W				
	(5)	subpopulation 3: 10 feet from the Portage Panorama sign	60°46'N	148°50'W				
	(5)	subpopulation 4: 50 feet from the Portage Panorama sign	60°46'N	148°50'W				
	(5)	subpopulation 5: 60 feet from the Portage Panorama sign	60°46'N	148°50'W				
	(5)	subpopulation 6: near top step of a different stairway	60°47"00"N	148°50'34"W				
	4	**Chichagof Island**, 50 miles NW of Sitka, AK. River Valley about Rust Lake.	57.6795 N	135.9567 W	970 ft	stream bank	22 July 2004	Kelly Calhoun, 199 & Brad Krieckhaus, 271

Table 2. Isozyme variation in *Arnica lessingii*. N* = average number of samples resolved per locus. P = percent polymorphic loci. A = average number of alleles per locus. Ho = observed heterozygosity. He = expected heterozygosity. Observed heterozygosity is very low compared to expected because the most common rare allele is a null; its heterozygotes could not be detected, but were presumably classified as homozygous for the common, active allele. Standard errors in parentheses.

Population	Location	N*	P	A	Ho	He
A. lessingii ssp. *lessingii*	Baranof Island	6.0 (0.0)	0	1.0 (0.0)	0	0.0
A. lessingii ssp. *norbergii*	Harlequin Trail	30.0 (0.0)	0	1.0 (0.0)	0	0.0
A. lessingii ssp. *norbergii*	Harlequin Lake	30.0 (0.0)	16.7	1.2 (0.1)	0.006 (0.006)	0.060 (0.041)
A. lessingii ssp. *norbergii*	Portage area	29.6 (0.4)	8.3	1.1 (0.1)	0	0.036 (0.036)
A. lessingii ssp. *norbergii*	Chichagof Island	4.0 (0.0)	16.7	1.2 (0.1)	0	0.071 (0.048)

Table 3. Genetic similarities among *Arnica lessingii* populations.

		Baranof Island	Harlequin Trail	Harlequin Lake	Portage area	Chichagof Island
ssp. *lessingii*	Baranof Island	-----				
ssp. *norbergii*	Harlequin Trail	1.000	-----			
ssp. *norbergii*	Harlequin Lake	0.991	0.991	-----		
ssp. *norbergii*	Portage area	0.993	0.993	0.997	-----	
ssp. *norbergii*	Chichagof Island	0.995	0.995	1.000	0.999	-----

Table 4. Frequencies of rare alleles in five *Arnica lessingii* populations. The estimate of the frequency of the rare UGPP allele is probably an underestimate; it is a null allele, and therefore heterozygotes were probably classified as the common, active allele.

Population	Location	IDH	UGPP
A. lessingii ssp. *lessingii*	Baranof Island	0	0
A. lessingii ssp. *norbergii*	Harlequin Trail	0	0
A. lessingii ssp. *norbergii*	Harlequin Lake	0.200	0.267
A. lessingii ssp. *norbergii*	Portage area	0	0.300
A. lessingii ssp. *norbergii*	Chichagof Island	0.250	0.250

Table 5. Wright's F-statistics for *Arnica lessingii* populations. These statistics calculated with all five populations included. If calculations are limited to the four *A. lessingii* ssp. *norbergii* populations, results are nearly the same.

Level 1		Level 2	F statistics	
Individuals	within	Population	Fis	0.965
Individuals	within	Total	Fit	0.969
Populations	within	Total	Fst	0.139

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Final Report

Genetic Variation in *Lupinus constancei:* Implications for Seed Transfer between Colonies

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MANAGEMENT SUMMARY

The pattern of isozyme variation in *Lupinus constancei* suggests that the two surviving colonies (Mt. Lassic colony and Red Lassic colony) are extremely similar genetically. Therefore, moving seeds or plants between the two colonies should not disrupt either population in regards to their genetic make-up as measured by isozymes.

INTRODUCTION

The Lassic Lupine, *Lupinus constancei* T. Nelson & J. Nelson, is a rare serpentine endemic found in California. Always rare, the plant has declined in recent years. Only two populations remain, totaling approximately 200 plants. One colony is tiny (Red Lassic colony from Humboldt County) and is on the verge of extirpation. The larger colony, Mt. Lassic colony, is also from Humboldt County.

Cross planting between the two surviving colonies could improve the probability that either or both will survive, both by boosting the number of plants in the colony and by introducing new genes that might overcome any inbreeding depression these small populations might suffer. On the other hand, cross planting might result in disrupting local coadapted gene complexes or otherwise harm the populations, if the two colonies have been separated long enough to evolve different adaptations.

Isozyme analysis was used to assess the amount of genetic differentiation between the two surviving *Lupinus constancei* populations. If the populations differ little, it is expected that cross planting will cause little or no harm to either population. If the populations are greatly differentiated, cross planting might be more harmful than beneficial. It should be noted, however, that isozymes are considered to be a neutral marker and therefore do not directly measure adaptation. The most definitive test regarding seed movement between colonies would involve a common garden experiment or reciprocal transplantation study to directly measure adaptive variation, and thus the consequences of seed movement.

METHODS

One leaf was collected from each of 24 plants (5 from the small Red Lassic colony and 19 from the larger Mt. Lassic colony). Leaves were shipped on ice to NFGEL and held refrigerated until they could be processed, within three days of arrival. Leaves were ground in liquid nitrogen, and approximately 0.5 ml Gottlieb buffer (USDA Forest Service 2003) was added to each sample. The resulting slurry was transferred to microtiter plate wells and frozen at –70ºC. On the day of electrophoresis, the extracts were thawed and absorbed onto 3 mm wide wicks prepared from Whatman 3MM chromatography paper.

Methods of electrophoresis followed the general methodology of Conkle et al. (1982), with some modifications (USDA Forest Service 2003). All enzymes were resolved on 11% starch gels. Four buffer systems were used for electrophoresis. A total of 23 loci were resolved well enough to score (Table 1).

Data were analyzed using BIOSYS-1, release 1.7 (Swofford and Selander 1989) and Popgene, version 1.21 (Yeh et al. 1997). A locus was considered polymorphic if an alternate allele occurred even once. We calculated unbiased genetic distances (Nei 1978), and expected heterozygosity (Nei 1973). The fixation indices were calculated by the method of Weir (1990).

RESULTS

Genetic Interpretation. Genetic interpretations were inferred directly from isozyme phenotypes based on knowledge of the generally conserved enzyme substructure, compartmentalization, and isozyme number in higher plants (Gottlieb 1981, 1982; Weeden and Wendel 1989). We are unaware of a chromosome count for *Lupinus constancei* (Goldblatt 2004), and therefore we scored it as diploid. Twenty of the loci yielded single, monomorphic bands on the gels: FEST, ACO, PGM, ME7, EST, PGI1, PGI2, LAP1, TPI1, TPI2, UGPP, CAT, MNR1, MNR2, IDH, G6PD, FDP, MDH1, MDH2, and 6PGD2. The remaining three loci (LAP2, 6PGD1, and DIA) yielded a diploid-like pattern, possibly showing hints of gene duplication or polyploidy. However, because there was not enough evidence of clear duplication/polyploidy at these three loci, they were scored like a diploid.

Genetic Diversity. *Lupinus constancei* has very low levels of variation, with only three (13%) polymorphic loci, and no more than two alleles per locus (Tables 2, 3). The two colonies were extremely similar, with variation in the same three loci (DIA, LAP2, and 6PGD1), and the same alleles at each of these loci (Tables 2, 3). Therefore, genetic identity between the two colonies was 0.999 and differentiation among colonies was a small component of total variation (Fst = 0.05).

DISCUSSION

The two *Lupinus constancei* populations are very similar as measured by isozymes. They showed the same kind of variation at the same loci. Because they are so similar, there appears to be no reason not to move plants between the two populations.

When making management decisions based on isozyme analysis, the strengths and limits of this procedure must be considered. The proteins assayed are basic metabolic enzymes coded for by alleles that may change more slowly than others involved with adaptations to particular sites. Therefore, some uncertainty always remains about the implications of isozyme analysis for adaptation. However, the extreme isozyme similarity between the two colonies (genetic similarity of 0.999) does imply that they have been isolated from each other for only a short time, and therefore they are unlikely to have differentiated significantly in any genes. Although absolute certainty is impossible, evidence supporting plans to exchange plants between the colonies is very strong.

LITERATURE CITED

Conkle, M. T., Hodgskiss, P. D., Nunnally, L. B., and Hunter, S. C. 1982. Starch Gel Electrophoresis of Conifer Seeds: a Laboratory Manual. General Technical Report PSW-64. Pacific Southwest Forest and Range Experiment Station, Forest Service, U.S. Department of Agriculture. Berkeley, CA 18pp.

Goldblatt, P., ed. (website accessed 30 August 2004). Index to Plant Chromosome Numbers (IPCN). Missouri Botanical Garden website: http://mobot.mobot.org./W3T/search/ipcn.htm.

Gottlieb, L. D. 1981. Electrophoretic evidence and plant populations. Progress in Phytochemistry 7: 1-46.

Gottlieb, L. D. 1982. Conservation and duplication of isozymes in plants. Science 216: 373-380.

Nei, M. 1973. Analysis of gene diversity in subdivided Populations. Proceedings of the National Academy of Science USA 70(12): 3321-3323.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Swofford, D. L. and Selander, R. B. 1989. BIOSYS-1, a computer program for the analysis of allelic variation in population genetics and biochemical systematics, release 1.7 edition. Illinois Natural History Survey, Champaign, Ill.

USDA Forest Service. 2003. National Forest Genetic Electrophoresis Laboratory Standard Operating Procedures. NFGEL, Placerville, CA.

Weeden, N. F. and Wendel, J. F. 1989. Genetics of plant isozymes. pp. 46-72 *in* D. E. Soltis and P. S. Soltis, eds., Isozymes in Plant Biology. Dioscorides Press, Portland, OR.

Weir, B. S. 1990. Genetic Data Analysis. Sinauer Associates. Sunderland, MA.

Yeh, F. C., Yang, R.-C., and Boyle, T. 1997. Popgene Version 1.20. University of Alberta, Edmonton, Alberta.

Table 1. Enzymes / buffer combinations and number of loci scored in isozyme analysis of *Lupinus constancei*. Buffer recipes are from USDA Forest Service 2003. Enzyme systems with zero loci scored are a result of insufficient resolution of the proteins on the starch gels.

LB buffer		SB buffer		MC6 buffer		MC8 buffer	
Enzyme System	**# Loci Scored**	**Enzyme System**	**# Loci Scored**	**Enzyme System**	**# Loci Scored**	**Enzyme System**	**# Loci Scored**
ACO *aconitase*	1	AAT *aminotransferase*	0	DIA *diaphorase*	1	DIA *diaphorase*	0
EST *esterase*	2	CAT *catalase*	1	FDP *fructose-1,6-diphosphatase*	1	FDP *fructose-1,6-diphosphatase*	0
FEST *fluorescent esterase*	1	GDH *glutamate dehydrogenase*	0	G6PDH *glucose-6-phosphate dehydrogenase*	1	G6PDH *glucose-6-phosphate dehydrogenase*	0
LAP *leucine aminopeptidase*	2	GLYDH *glycerate-2-dehydrogenase*	0	IDH *isocitrate dehydrogenase*	1	IDH *isocitrate dehydrogenase*	0
ME7 *malic enzyme*	1	MNR *menadione reductase*	2	MDH *malate dehydrogenase*	0	MDH *malate dehydrogenase*	1
PGI *phosphogluco-isomerase*	2	TPI *triosephosphate isomerase*	2	6PGD *phosphogluconate dehydrogenase*	2	6PGD *phosphogluconate dehydrogenase*	0
PGM *phosphogluco-mutase*	1	UGPP *uridine diphosphoglucose pyrophosphorylase*	1	SKD *shikimic acid dehydrogenase*	0	SKD *shikimic acid dehydrogenase*	0

Table 2. Allele frequencies of variable loci in *Lupinus constancei*.

Enzyme	Locus	Red Lassic	Mt. Lassic
DIA	1	0.700	0.947
DIA	2	0.300	0.053
LAP2	1	0.700	0.500
LAP2	2	0.300	0.500
6PGD1	1	0.700	0.816
6PGD1	2	0.300	0.184

Table 3. Genetic variation in *Lupinus constancei* based on 23 isozyme loci treated as diploid. N = sample size. P = percent polymorphic loci. A = alleles per locus. Ae = effective alleles per locus (a measure of the evenness of allele frequencies). Ho = observed heterozygosity. He = expected heterozygosity. Standard error in parenthesis.

Population	N	P	A	Ae	Ho	He
Red Lassic colony	5	13%	1.13	1.09	0.078 (0.043)	0.061 (0.034)
Mt. Lassic colony	19	13%	1.13	1.07	0.041 (0.030)	0.040 (0.026)
All samples	24	13%	1.13	1.07	0.049 (0.150)	0.045 (0.128)

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Final Report

Taxonomic identification of *Erythronium* (Fawn-lily) samples from the Olympic Peninsula, Washington

© 2004 Dean Wm. Taylor
Erythronium revolutum

© Gary A. Monroe. Mount Rainier National Park, WA.
(Gary A. Monroe @ USDA-NRCS PLANTS Database)
Erythronium montanum

Contact: Valerie D. Hipkins, NFGEL Director
NFGEL Project #170

August 27, 2004

Submitted Problem Statement

The genus *Erythronium* is represented by three species on the southwest side of the Olympic Peninsula (Allen 2001). The Quinault Fawn-lily, *E. quinaultense* (ERQU), is a recently described "new" species, a tetraploid, and morphologically intermediate between *E. montanum* (ERMO) and *E. revolutum* (ERRE). It is listed as threatened on the Washington State Sensitive Species List and will undoubtedly be added to the Regional Forester's Special Species of Concern within the next six months. Unaware of this tetraploid species, all fawn-lily's located in mid- to low-elevations in the past were identified as ERRE. We suspect that ERQU is present in three units of the pending Matheny Timber Sale. All occurrences of both ERQU and ERRE on the Olympic NF need to be revisited and reidentified as to species. We would like to use ploidy analysis to verify species ID that has been based on morphology.

Submitted Management Implications

It is critical that we confirm species identification as the presence of ERQU will change the boundaries and harvest methods use in the Mathey Timber Sale. New range maps will be used in development of a management guide for ERQU. Also, the updated (smaller) range of ERRE may lead to the relisting of this species on the Washington State Sensitive Species List and the Regional Forester's Special Species of Concern.

Submitted Material

One to two leaves per individual were submitted from seven sites located on the Olympic National Forest for analysis following NFGEL Collection Guidelines (Table 1). Species identity was determined by morphology.

Table 1. Site locations and number of samples submitted for ploidy analysis.

SITE ID	SITE NAME	SITE LOCATION	SPECIES	# SAMPLES
QUIN-A	South Shore	T23N, R09W, S20, NE ¼, Elevation 60 m	ERRO	5
QUIN-B	Higley Peak	T23N, 10W, S01, SE ¼, Elevation 840 m	ERQU	8
QUIN-C	Finley	T24N, R10W, S24, NE ¼, Elevation 360 m	ERQU	8
QUIN-D	2140 Rd/B-18	T24N, 10W, S28, SW ¼, Elevation 750 m	ERQU	6
QUIN-E	2170-175	T24N, R10W, S14, NW, Elevation 850 m	ERQU	4
			ERMO	1
QUIN-F	2170-180	T24N, R09W, S07, SW, Elevation 925 m	ERMO	6
QUIN-G	2170-310	T24N, R09W, S09, SW, Elevation 925 m	ERMO	6
Total				*44*

Laboratory Methods

ISOZYMES: In order to make a cursory examination of isozyme activity in this genus, six samples were prepared for analysis by placing one hole punch of leaf tissue per individual in two drops (plastic) of Melody/Neale buffer following NFGEL Standard Operating Procedures. Samples were run on three buffer systems using 11 enzyme stains (*LB:* ME7, PGI, LAP, PGM; *SB:* UGPP, TPI, AAT, 6PGD; *MC6:* MDH, EST, IDH).

DNA: DNA was extracted from the 44 samples for possible future use following the Qiagen DNEasy-96 liquid nitrogen protocol.

PLOIDY: Approximately 50mg of leaf tissue per individual was macerated in 0.5ml extraction buffer (as per the Partec 2-step staining protocol) with a sharp razor blade in a small petri dish. The sample incubated at room temperature for 2.5 hours in the dark, after which 1.5ml staining solution was added per sample. The solution was filtered through a green celtrics filter, and read on a Partec PA-1 using the following settings: lg2 scale, 200 gain, 10 L-L, and speed = 1. Extra leaf material was frozen at -80C to use as an internal control. These internal controls were used throughout the analysis, and peak position was corrected by run date.

Results and Discussion

ISOZYMES: The isozyme analysis showed strong enzyme activity in seven on the 11 stains, moderate activity in three of the stains (6PGD, TPI, and MDH), and poor activity in only one stain (PGI). PGI activity could likely be improved with a change to the sample preparation method, gel position, or gel buffer type. Overall, the results indicate that *Erythronium* is very amenable to isozyme analysis, and this type of data would be beneficial at studying levels and patterns of genetic diversity within the genus.

DNA: Sufficient yields of high molecular-weight DNA were obtained per sample (yields generally exceeded 4ug per sample). DNA is frozen at -80C for any future use.

PLOIDY: The Quinault Fawn-lily (*E. quinaultense*) is a recently described tetraploid species from the Olympic Mountains in the state of Washington. It is a morphological intermediate between two diploid species found in that area, *E. montanum* (Avalanche Fawn-lily) and *E. revolutum* (Pink Fawn-lily). It is thought that the distinct Quinault Fawn-lily species is derived from hybridization between these two diploid *Erythronium* species (Allen 2001).

We used flow cytometry to determine species identity through sample ploidy levels. Quite simply, if a sample of *Erythronium* from the Olympic Mountains is tetraploid, its species identity is *E. quinaultense*. A diploid sample collected from that area can be either *E. montanum* or *E. revolutum*.

Among the 44 samples submitted for analysis, two ploidy levels were detected (Figure). A sample with ploidy level #1 was represented by a peak at position 15 in the flow cytometry output. A sample with the second ploidy level was represented by a peak at position 30. A sample whose ploidy data is '30', contains twice as much DNA as a sample whose ploidy data is '15'. Therefore, if the peak data of '15' corresponds to diploid plants, the data of '30' corresponds to double that, or to a tetraploid.

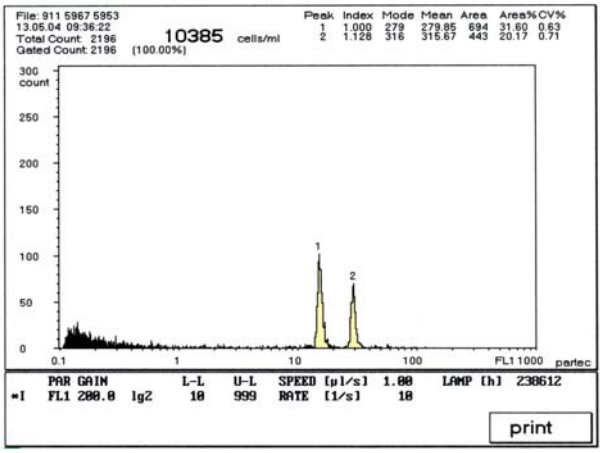

Figure. Flow cytometry output using a Partec PA-1 ploidy analyzer. This shows the ploidy results from two samples run simultaneously. Peak #1 is from sample QUIN-A3 (*E. revolutum*), and peak #2 is from sample QUIN-C4 (*E. quinaultense*). QUIN-A3 has a peak position of '15' and is the diploid; QUIN-C4 has a peak position of '30' and is the tetraploid.

Seventeen of the 44 samples analyzed are diploid, the remaining 27 plants are tetraploid (Table 2). Ploidy results show that three of the sites, 'South Shore', '2170-180', and '2170-310' contain all diploid plants. These sites were thought to contain the species *E. revolutum* (in 'South Shore') and *E. montanum* (in '2170-180' and '2170-310'). Ploidy analysis also indicates that all samples from sites 'Higley Peak', 'Finley', '2140 Rd/B-19', and '2170-175' are tetraploid. All these plants were thought to be *E. quinaultense* (the tetraploid species), except for sample QUIN-E3, which was thought to be *E. montanum*. The ploidy level of QUIN-E3 is tetraploid, however, indicating that it is not *E. montanum*, but instead *E. quinaultense*.

Table 2. Site locations and ploidy results by individual.

SITE NAME	SPECIES ID	SAMPLE ID	Ploidy Results	
			Peak Position	Ploidy Level
South Shore	*E. revolutum*	QUIN-A1 thru A5	15	Diploid
Higley Peak	*E. quinaultense*	QUIN-B1 thru B8	30	Tetraploid
Finley	*E. quinaultense*	QUIN-C1 thru C8	30	Tetraploid
2140 Rd/B-19	*E. quinaultense*	QUIN-D1 thru D6	30	Tetraploid
2170-175	*E. quinaultense*	QUIN-E1 thru E2	30	Tetraploid
	E. montanum	QUIN-E3	30	Tetraploid
	E. quinaultense	QUIN-E4 thru E5	30	Tetraploid
2170-180	*E. montanum*	QUIN-F1 thru F6	15	Diploid
2170-310	*E. montanum*	QUIN-G1 thru G6	15	Diploid

Conclusion

Because Allen (2001) determined that *E. quinaultense* in tetraploid, while *E. revolutum* and *E. montanum* are diploid, we can use the ploidy results to make a species ID on material located on the Olympic National Forest.

Ploidy analysis confirmed the presence of:

- tetraploid *Erythronium* (therefore, *E. quinaultense*) from four sites located on the Olympic National Forest ('Higley Peak', 'Finley', '2140 Rd/B-19', and '2170-175'), and
- diploid *Erythronium* (therefore, either *E. revolutum* or *E. montanum*) from three sites, 'South Shore', '2170-180', and '2170-310', also from the Olympic National Forest.

Sample QUIN-E3 was submitted as *E. montanum* (from site '2170-175'). However, ploidy data indicate it is tetraploid, not diploid, therefore identifying it as *E. quinaultense*.

Reference

Allen, G.A. 2001. Hybrid speciation in *Erythronium* (Liliaceae): a new allotetraploid species from Washington State. Systematic Botany 26(2):263-272.

STAFF ACTIVITIES

Meetings, Shortcourses, and Workshops

Presentations

2003. V. Hipkins. Molecular approaches to restoration issues. USDA Forest Service, Ecological Genetics and Native Plant Material Development meeting. Portland, OR, November 3 – 5.

2003. V. Hipkins. Genetics in silviculture: a Regional perspective. USDA Forest Service, Region 8 Silviculture Meeting. Cadiz, KY, Nov 17 – 19.

2004. V. Hipkins. Genetic diversity in Monterey Pine on California Highway 1 near Carmel, Monterey County. Pitch Canker Task Force, Annual Research Meeting, Univ. of California. Davis, CA, February 9.

2004. V. Hipkins, J. Kitzmiller, David Burton, and Don Yasuda. Joint Presentation. Genetics and the distribution of trembling aspen clones in the Central Sierra Nevada, California. Wildlife Society Meeting, Rohnert Park, CA, February 25 – 26.

2004. V. Hipkins. Tomorrow's applied answers in today's basic science. USDA Forest Service, Joint Meeting with the Pacific Southwest Research Station Leadership Team and the Regional Leadership Forum. Sacramento, CA. March 9.

2004. A. Groover and V. Hipkins. Tomorrow's applied conservation and management answers in today's basic science. USDA Forest Service, Meeting with the Pacific Southwest Research Station Leadership Team and the Associate Chief, Sally Collins. Sacramento, CA. March 10.

2004. V. Hipkins. Applied genetics at NFGEL. USDA Forest Service, Institute of Forest Genetics Technical Advisory Visit. Placerville, CA. March 17.

2004. R. Schmidtling, V. Hipkins, and C.D. Nelson. Comparing genetic variability of an American pine species (*P. elliottii*) with a Chinese pine species (*P. massoniana*). N. Amer. For. Biol. Workshop, Michigan Technological University, Houghton, Michigan, July 12-15.

2004. V. Hipkins. NFGEL Steering Committee Meeting. Placerville, CA. April 29.

2004. V. Hipkins. Genetics in botany. ('Consumnes Lecture'). Folsom, CA. May 10.

2004. V. Hipkins. Genetics in Silviculture. R-9 Silviculture Meeting. Bartlett, New Hampshire. June 7 – 11.

2004. V. Hipkins. Assessing Pollen Contamination in Douglas-fir. Pacific Northwest Tree Improvement Research Cooperative, Annual Meeting. Salem, OR. June 29.

2004. V. Hipkins. NFGEL: Applied conservation management in today's basic science. Forest Service Washington Office. Washington DC. August 11.

2004. M. Maldonado and V. Hipkins. National Forest System Genetic Resource Program Briefing to the Acting Director of Forest Management. Forest Service Washington Office. Washington DC. August 11.

Attended

2004. V. Hipkins. Visit to Appalachia HIDA Signature Laboratory. Lexington, KY, January 21 – 23.

2004. Robert Saich. Molecular Markers *ICBR* Workshop. University of Florida, Gainesville, FL . March 1 – March 5.

2004. Ricardo Hernandez. Visit to Colegio de Postgraduados, Instituto do Recursos Naturales to provide training in isozyme techniques. Montecillo, Texcoco, Mexico.

March 13 – March 21.

2004. Jennifer DeWoody. 2nd - 40 Supervisor Training. Lake Tahoe, Nevada. March 22 - March 26.

2004. Jennifer DeWoody. Recent Advances in Conservation Genetics. Front Royal, VA. August 16 - August 27.

2004. V. Hipkins. Office of National Drug Control Policy (ONDCP). Meeting regarding DNA Fingerprinting of marijuana. Washington DC. August 10.

Meetings Hosted

2004. Hosted USDA Forest Service, National Genetics Meeting. Institute of Forest Genetics, Placerville, CA. April 27 – 29.

Internal Activities

Member of the National Forest Service Safety Committee (R Meyer)
Union President – Pacific Southwest Research Station (R Meyer)

Hosted

NFGEL continues to host a variety of visitors. Tours of the facility and operation were provided to Forest Service employees, members of the public and private industry, university faculty and classes, foreign scientists, and employees from other state and federal government agencies.

Collaborations and Cooperations

NFGEL formed collaborations with FS Research Stations, Bureau of Land Management, California Department of Transportation, US Fish and Wildlife Service, University of California at Davis, private companies, and non-profit groups. We hosted local high-school students on a volunteer basis. We also collaborate internally within the Agency to lend expertise in the area of genetics.

STAFFING

During FY04 (10/1/03 to 10/1/04), NFGEL was staffed with three permanent full-time, two TERM, six temporary employees, and two high-school senior volunteers.

Name	Position	Tour	E-mail Address
Valerie Hipkins	Director	PFT	vhipkins@fs.fed.us
Jennifer DeWoody	Lab Manager/Biologist	TERM	jdewoody@fs.fed.us
Pat Guge	Lab Biotechnician	PFT	pguge@fs.fed.us
Randy Meyer	Lab Biotechnician	PFT	rmeyer@fs.fed.us
Robert Saich	Lab Biotechnician	TERM	rcsaich@fs.fed.us
Robert Westfall	Scientist	Coop	rwestfall@fs.fed.us
Ricardo Hernandez	Lab Biotechnician	Temp	ricardohernandez@fs.fed.us
Ashley Lindstrom	Lab Biotechnician	Temp	alindstrom@fs.fed.us
Wesley Calidonna	Lab Biotechnician	Temp (7/04 – present)	wcalidonna@fs.fed.us
Jens Hamar	Lab Biotechnician	Temp (11/03 – 4/04)	--
Bethany Hynan	Lab Biotechnician	Temp (6/04 – 8/04)	--
Bernardo Ortiz	Lab Biotechnician	Temp (10/03 – 6/04)	--
Lars Rockholm	Lab Biotechnician	Volunteer (10/03 – 5/04)	--
Kenneth Choi	Lab Biotechnician	Volunteer (10/03 – 5/04)	--

BUDGET

Activity	FY03	FY04
Receipts (in thousands)		
Allocation	378.0	410.0
Carryover	52.0	0.0
Soft Money, after indirect removed	233.5	268.8
-Fire Transfer	-30.0	27.8
Total	633.5	706.6
Expenditures (in thousands)		
Salary (permanant)	*201	**273.3
(temperary)	80.9	54.3
Overhead to Headquarters	40.0	42.0
Overhead to Site	38.2	41.9
Chemicals/Supplies	77.0	48.2
Equipment	97.6	189.9
Travel/Training	7.8	12.5
Awards	2.0	1.3
Books/subscriptions	0.4	0.1
Computers (not including FOR)	18.9	1.0
Repair	4.7	2.9
Photos/Slides/Publications	1.6	27.4
Postage	0.7	0.1
Office Supplies	0.7	0.6
Furniture	2.1	3.0
Total	573.6	698.5
Balance	59.9	8.1

* does not include $17.9 in salary due to alternate salary sources
** does not include $24.1 in salary due to alternate salary sources

FY 04 Soft Money

Source	Amount ($)	Percentage
FS-NFP (WO)	183.2	68.2%
FS-R6	6.6	2.5%
FSR-RMRS	30.0	11.2%
FSR-PSW	4.5	1.7%
USFWS	4.1	1.5%
NPS	20.5	7.6%
CA Dept of Transport.	9.0	3.3%
Private Companies	10.9	4.0%
Total	268.8	100.0%

Project Workload, FY04

ISOZYMES (starch gel electrophoresis)

By Project

Reagion or Agency	Project#	Species	# gels	# run days	# weeks
R-2	103	*Pinus ponderosa*	28.0	4.0	2.00
RMRS	104	*Viquiera multiflora*	6.0	1.0	0.50
RMRS	110	*Astragalus utahensis*	3.0	0.5	0.25
RMRS	115	*Lupinus argentus*	3.0	0.5	0.25
R-6/BLM	125	*Peseudotsuga menziesii*	111.0	17.0	8.00
RMRS	132	*Atriplex canescens*	9.0	2.0	1.00
RMRS	139	*Artemisia tridentata*	9.0	1.0	0.50
USFWS	144	*Erysimum*	23.0	4.0	2.00
RMRS	152	*Crepis occidentalis*	3.0	0.5	0.25
RMRS	153	*Purshia tridentata*	17.0	3.0	1.50
R-9	155	*Pinus stobus*	172.0	27.0	13.00
NPS	158	*Oenothera wolfii*	60.0	10.0	5.00
R-6	161	*Chamaecypaeis lawsoniana*	1.0	0.5	0.25
USFWS	163	*Lupinus constance*	7.0	2.0	1.00
R-6	164	*Pinus albicaulis/P.monticola*	18.0	2.0	1.00
R-6	183	*Chamaecypaeis lawsoniana*	3.0	0.5	0.50
PSW	188	*Picea chihuahuana*	11.0	2.0	1.00
NFGEL		starch testing	10.0	2.0	1.00
NFGEL		testing tissue for preps	18.0	5.0	2.50
TOTAL			**512.0**	**84.5**	**41.5**

ISOZYMES (starch gel electrophoresis) continued

By Forest Service Region or Agency

Region or Agency			#gels	#days	#weeks
Forest Service					
	National Forest System				
		R-2	28.0	4.0	2.00
		R-6	22.0	3.0	1.75
		R-6/BLM	111.0	17.0	8.00
		R-9	172.0	27.0	13.00
		NFGEL	28.0	7.0	3.50
	Research				
		RMRS	50.0	8.5	4.25
		PSW	11.0	2.0	1.00
USFWS			30.0	6.0	3.00
NPS			60.0	10.0	5.00

R = Region
RMRS = Rocky Mountain Research Station
USFWS = United Stated Fish and Wildlife Service
BLM = Bureau of Land Management
NPS=National Park Service

DNA

By Project

Region or Agency	Project #	Species	# DNA Extractions	Extraction Method	# PCR Reactions	# ABI capillary runs	# employee hours
FS-NFS-R2	103	*Pinus ponderosa*	170	DNEasy-96	2466	105	429
			3	DNEasy-Maxi			
FS-FSR-RMRS	114	*Erigonum umbellatum*	81	DNEasy-96			10.1
FS-FSR-RMRS	116	*Lupinus sericious*	9	DNEasy-Mini			4.7
			19	DNEasy-96			
FS-NFS-R6 /BLM	125	Douglas-fir	161	DNEasy-96			20.2
FS-FSR-RMRS	133	*Atriplex spp.*	1	DNEasy-Mini			11.2
			87	DNEasy-96			
FS-FSR-RMRS	140	*Artemesia tridentate*	40	DNEasy-96			5
FS-FSR-RMRS	142	*Chrysothamnus nauseosus*	64	DNEasy-96			8
FS-NFS-R8,R9	147	Eastern grasses	392	DNEasy-96			49
FS-NFS-R5	150	*Populus tremuloides*	11	DNEasy-Mini	4174	147	450
			850	DNEasy-96			
FS-FSR-RMRS	151	*Balsamorhiza sagittata*	20	DNEasy-96			2.5
FS-FSR-RMRS	153	*Purshia tridentata*	20	DNEasy-96			17.4
			45	FastPrep Bio-101			
FS-NFS-R9	155	*eastern white pine*	503	DNEasy-96	1748	65	208
NPS	156	Whitebark pine	3	DNEasy-Mini			46.2
			363	DNEasy-96			
FS-NFS-R6	157	Whitebark pine	423	DNEasy-96			52.9
Univ Calif	165	Monterey pine	864	DNEasy-96			108
FS-NFS-R8	168	Virginia/shortleaf pines	33	DNEasy-96	146	5	50

By Project cont...

Private	169	*Populus* spp.	7	DNEasy-96	192	4	15.8
			7	DNEasy-Mini			
FS-NFS-R6	170	Pink fawn lily	27	DNEasy-96			3.4
FS-NFS-R5	171	*Vaccinium* spp.	25	DNEasy-Mini			12.4
			49	DNEasy-96			
FS-NFS-R6	173	Port-Orford Cedar	4	DNEasy-Mini			54.9
			431	DNEasy-96			
FS-FSR-RMRS	175	*Lomatium* spp.	6	DNEasy-Mini			14
			100	DNEasy-96			
FS-FSR-RMRS	176	*Phlox longifolia*	2	DNEasy-Mini			8.4
			63	DNEasy-96			
FS-FSR-RMRS	177	*Tragopogon dubius*	61	DNEasy-96			7.7
FS-FSR-RMRS	178	*Penstemon deustus*	26	DNEasy-Mini			36.5
			121	DNEasy-96			
			45	FastPrep Bio-101			
FS-FSR-RMRS	179	*Ceratoides*	2	DNEasy-Mini			10.7
			81	DNEasy-96			
FS-NFS-R9	180	Yew hybrids	13	DNEasy-96			1.7
USFWS	181	Western Lily	18	DNEasy-96			2.3
Private	182	*Populus* spp.	408	DNEasy-96			51

DNA

By Region or Agency

Region or Agency	# DNA Extractions	# PCR Reactions	# ABI runs	# employee hours
FS-NFS-R2	173	2466	105	429
FS-NFS-R5	935	4174	147	462.4
FS-NFS-R6	885			111.2
FS-NFS-R6 /BLM	161			20.2
FS-NFS-R8	33	146	5	50
FS-NFS-R9	503	1748	65	208
FS-NFS-R8/R9	392			49
FS-FSR-RMRS	893			136.2
USFWS	18			2.3
NPS	366			46.2
University of California	864			108
Private	422	192	4	66.8
TOTAL	5645	8726	1368	1635.3

FS=Forest Service
FSR=Forest Service Research
RMRS=Rocky Mountain Research Station
NFS=National Forest System
R#=Region Number
Private=Private Company
BLM=Bureau of Land Management
USFWS=US Fish and Wildlife Service
NPS=National Park Service