This is to certify that the

dissertation entitled

Isozyme Analysis of the Blue-Engelmann Spruce Complex in Southwestern Colorado

presented by

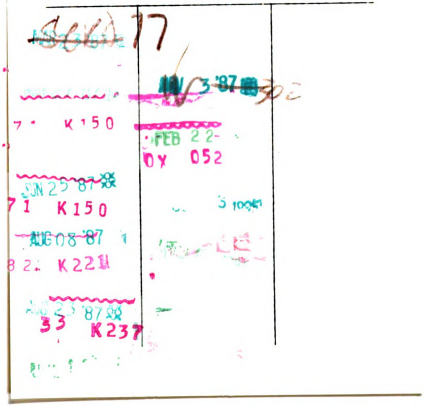
Stephen Gerard Ernst

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Forest Genetics

Major professor

Date Sept. 26, 1985



ISOZYME ANALYSIS OF THE BLUE-ENGELMANN SPRUCE COMPLEX IN SOUTHWESTERN COLORADO

By

Stephen Gerard Ernst

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Forestry

1985

ABSTRACT

ISOZYME ANALYSIS OF THE BLUE-ENGELMANN SPRUCE COMPLEX IN SOUTHWESTERN COLORADO

By

Stephen Gerard Ernst

A partial diallel mating design incorporating 20 parents each of blue and Engelmann spruce in the Dolores River drainage of southwestern Colorado was utilized to generate intraspecific and interspecific full-sib progeny. Percent germinated and percent ungerminated-but-full seed on a total seed basis were used as measures of crossability and abnormal seed production, respectively.

Production of viable intraspecific seed was primarily under the control of nonadditive sources of genetic variation in both blue and Engelmann spruce. Maternal effects were also very important in the production of viable intraspecific seed in blue spruce, but not in Engelmann spruce. Interspecific crosses were successful only with Engelmann spruce as the female parent; no viable seed were produced from the reciprocal species cross. Production of viable interspecific seed with Engelmann spruce as the female parent was primarily under the control of additive sources of genetic variation, but control by nonadditive sources of variation was also evident.

The inheritance of isozymes representing 13 loci from 11 enzyme systems was determined in both blue and Engelmann

spruce. The isozymic composition of the two species was quite different in the Dolores River drainage, and several species-specific alleles and strong frequency differences were observed at several loci. Positive identification of control-pollinated interspecific hybrids was achieved using isozyme analysis.

No natural F_1 hybrids were observed in the field among sampled mature trees based on isozyme analysis, nor among open-pollinated embryos from mother trees located at a site where both blue and Engelmann spruce are present and pollen shed and female strobilus receptivity are coincident between the two species in the spring. However, based on the low crossability between the two species, if natural hybridization does occur between blue and Engelmann spruce it must be of very low frequency and beyond the resolution of this study based on the number of enzyme loci and the number of individuals sampled. Therefore, if natural hybrids do exist and are fertile, evidence for introgression would be rapidly diluted by backcrossing.

ACKNOWLEDGEMENTS

I would like to thank Dr. J.W. Hanover (Chairman) for the support, patience, trust and freedom he has extended to me during my tenure at Michigan State University. I would also like to thank the members of my guidance committee: Dr. D.E. Keathley for his encouragement, advise and open door; Dr. I.L. Mao for his generous help in the statistical analysis; and Dr. J. Hancock for his help in presenting the results of the isozyme analysis. Also, the help of many other members of the Department of Forestry, including staff members, fellow graduate students and work-study students, is much appreciated.

To my parents, I could never thank them enough for all they have given and imparted to me, and I hope my work is a favorable reflection on their efforts and love. Also, I would like to thank my fiancee, Jane, for the encouragement only a water ouzel can bring.

TABLE OF CONTENTS

LIST OF TABLES

LSIT OF FIGURES

CHAPTER I

INTRODUCTION

A biological species has been defined as a group of natural populations which can actually or potentially interbreed but are reproductively isolated from other such groups (Ayala 1982). Reproductive isolation is imperative for the genetic divergence of two populations into distinct species because gene frequency changes in one population cannot then be incorporated into the other through interbreeding. Two species can be reproductively isolated either through geographical separation or as a result of internal genetic mechanisms. When reproductively isolated, each species represents an independent and discrete evolutionary unit.

Mechanisms of reproductive isolation have been grouped into two classifications based on the occurrence and success of interspecific matings--prezygotic reproductive isolating mechanisms which prevent the formation of interspecific zygotes, and postzygotic mechanisms which reduce the viability or fertility of interspecific hybrids (Ayala 1982; Mayr 1963). Prezygotic isolating mechanisms include: seasonal (allochronic) and habitat (allopatric) isolation such that potential mates do not meet; behavioral (ethological) isolation where potential mates meet but do not mate; mechanical isolation where transfer of gametes is

attempted but is not successful; and gametic isolation where sperm or pollen is transferred but the egg is not fertilized. Postzygotic isolating mechanisms include hybrid inviability where hybrid zygotes die or fail to reach sexual maturity; hybrid sterility where the hybrid zygotes are viable but partially or completely sterile; and hybrid breakdown where the F_2 or backcross hybrid progeny have reduced viability or fertility. Prezygotic mechanisms are considered to be more efficient evolutionarily because they reduce unneeded expenditure of limited resources in the development of nonviable or infertile progeny (Grant 1981).

Reproductive isolation can occur as a by-product of evolutionary divergence between populations or species, or by direct selection for these mechanisms. If two populations become separated geographically, they may accumulate enough divergent characters independently to be considered separate races or species. If the two populations then come into sympatry (naturally or artificially), they may or may not be capable or producing viable hybrid progeny, depending on the degree and types of genetic divergence. If the two groups became genetically reproductively isolated during separation, these mechanisms may have been the result of pleiotropic effects associated with other genetic changes rather than direct selection for them. Conversely, the two groups may be capable of

interbreeding, with geographic separation the only cause for their independent genetic integrities. Many examples of these two scenarios occur in nature (see Futuyma 1979).

While geographic isolation is regarded as the most common mode of speciation (Mayr 1963; Lewontin 1974; Grant 1981), putative examples of direct selection for reproductive isolation do exist in nature (Futuyma 1979; Grant 1981). Grant (1981) has given five special conditions for selection to enhance hybridization barriers in plants. First, the races or species must be sympatric in distribution. Second, hybridization must be deleterious, where the hybrid progeny are less viable or infertile. Third, hybridization results in a loss of reproducitve potential to the two groups, and that loss is disadvantageous. Fourth, absolute incompatibility may not necessarily result, the extent of hybridization dependent on how effectively the hybrids are eliminated by natural selection. Lastly, only those isolating mechanisms which are manifest in the parental generation will respond effectively to selection for isolation.

The genus Picea (spruce) presents some interesting examples of reproductive isolation and speciation. Wright (1955) observed that Picea species which were the most widely separated geographically and the most divergent morhpologically generally crossed with the least success. However, there do exist species combinations in Picea which occupy very similar geographic distributions and are

morphologically quite similar, but still possess very strong hybridization barriers; e.g., among the North American spruces, white and black spruce (P. glauca (Moench) Voss. and P. mariana (Mill.) B.S.P., respectively), and blue and Engelmann spruce (P. pungens Engelm. and P. engelmannii Parry ex Engelm., respectively). These species combinations may represent instances of selection for reproductive isolation. Whether reproductive isolation in such species combinations resulted as a by-product of geographic (allopatric) speciation and was enhanced when the two species came into sympatry, or occurred while in sympatry and caused their evolutionary divergence into distinct species (sympatric speciation) can only be speculated until further study.

There is considerable overlap in the ranges of blue and Engelmann spruce, with the range of blue spruce contained almost entirely within that of Engelmann spruce. They are both montane species native to the Rocky Mountain region of North America, but occupy somewhat different habitats. Blue spruce is generally found in the semiarid riparian habitats of the montane zone at elevations of 2000 to 3000 meters. Associated species include trembling aspen (Populus tremuloides Michx.), ponderosa pine (Pinus ponderosa Dougl. ex Laws.) and Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco). Engelmann spruce generally occurs above 3000 meters along the upper valleys and

hillsides in pure stands or mixed with subalpine fir (Abies lasiocarpa (Hook.) Nutt.). In the elevationally intermediate zones both species are often present in close proximity, with ample opportunity for cross-pollination.

Blue and Engelmann spruce are quite similar morphologically, but can be distinguished using a combination of traits (Daubenmire 1972; Jones and Bernard 1977; Schaefer and Hanover 1985a). The most diagnostic characters are usually associated with female cone morphology, bark texture, twig pubescence, shape of bud scales and needle sharpness (Mitton and Andalora 1981). Chemical traits have also been effective in distinguishing the two species (Taylor et al. 1975; Schaefer and Hanover 1985b). However, there is enough overlap in morphological and biochemical traits between between blue and Engelmann spruce to suggest either introgression or coadaptation between the two species (Daubenmire 1972; Taylor et al. 1975; Mitton and Andalora 1981). Schaefer and Hanover (1985c) combined morphological and terpene data using discriminant analysis and observed intermediacy between the two species in southwestern Colorado.

Artificial hybrids between blue and Engelmann spruce have been produced, but viable seed yields from the interspecific crosses were very low. Fechner and Clark (1969) obtained two percent viable hybrid seed for this interspecific cross, but only with Engelmann spruce as the female parent; the reciprocal cross did not produce any

viable seed. Kossuth and Fechner (1973) obtained less than one percent viable hybrid seed, but only with blue spruce as the female parent; the reciprocal cross was not successful. However, in each of these studies only one or two parents of each species were used to make the controlled crosses. The selection of parents was shown to influence seed yields in both intraspecific (King et al. 1970) and interspecific (Kudray and Hanover 1980) crosses in spruce. Therefore, a large number of parents is required to accurately quantify interspecific crossability in this genus. It is reasonable to assume, based on previous morphological, chemical and crossability studies that the interspecific crossability between blue and Engelmann spruce is very low.

In the elevationally intermediate zones where both blue and Engelmann spruce are present, there is enough overlap between the phenology of pollen shed and female strobilus receptivity of these two species for natural cross-pollination to occur (Fechner and Clark 1969; Ernst, unpublished data). Therefore allopatric or allochronic prezygotic reproductive isolating mechanisms are not isolating factors at present. Nor are ethological mechanisms, as the spruces are anemophilous. Kossuth and Fechner (1973) did document very clearly that gametic isolation is manifest in this interspecific cross. Normal development of the female gametophyte apparently requires

some type of stimulus from the intraspecific pollen. However, the incompatibility mechanism between these two species is more complex than this, as breakdown was observed at several stages of ovule and embryo development, and crossability barriers were not complete (Kossuth and Fechner 1973). Hybrid inviability has also been observed among blue-Engelmann hybrid embryos and seedlings (Fechner and Clark 1969; Kossuth and Fechner 1973). Unfortunately, the hybrids generated in the two studies mentioned were destroyed in a greenhouse fire (Fechner, personal communication), and no known mature hybrids exist to assess hybrid fertility in the F_1 and later generations.

Blue and Engelmann spruce are believed to be closely related phylogenetically, blue spruce possibly the result of a single speciation event from the progenitor Engelmann spruce (Nienstaedt and Teich 1971; Daubenmire 1972; Taylor et al. 1975). Blue spruce may have evolved from an ecotype of Engelmann spruce which was adapted to the semiarid lowlands and was partially (sympatrically or parapatrically) or completely (allopatrically) geographically isolated. Taylor et al. (1975) suggested the two species resulted from a geographical (allopatric) speciation event, and incompatibility was a by-product of evolutionary divergence. When the two species came into sympatry, the hybridization barriers may not have been as strong as they are today, but reproductive isolation was advantageous due to habitat requirements of the two

incipient species and thus was enhanced to its present state. Whether or not hybrid inviability or infertility also had an effect cannot be determined until known hybrids are observed through maturity and F_2 crosses are attempted. Hybrid inviability or infertility seems essential if strong incompatibility barriers were not present when the two incipient species came into sympatry.

Another possibility is that blue and Engelmann spruce speciated while in sympatry. At least two different scenarios can be envisioned for such an event. A quantum speciation event may have occurred which resulted in a race or species much better adapted to the more arid environment now occupied by blue spruce. Again, reproductive isolation may have been advantageous because of ecological divergence between the two incipient species, or possibly the same mutational event made the incipient species incompatibile with Engelmann spruce but compatible with itself. Another scenario is where a mutation occurred in a marginal population of Engelmann spruce--located in a more arid environment--which rendered it at least partially incompatible with Engelmann spruce but compatible with itself. The ensuing population then exploited the more arid habitat and incompatibility was further enhanced due to niche separation and hybrid inviability.

All speculation aside, it is necessary to first quantify the genetic variation in blue and Engelmann spruce

across their respective ranges and determine how the environment influences the expression of those traits before any speciation theories can be adequately evaluated. Also, the genetic variability and control of intraspecific and interspecific crossability and its physiological basis must be quantified using a large number of parents from throughout the respective ranges of the two species. Morphological, biochemical and physiological traits need to be quantified in common garden experiments and the degree of genotype x environment interaction assessed. Also, a technique which will accurately identify interspecific hybrids needs to be developed.

The objectives of this study were to (1) assess the genetic variation and control of intraspecific and interspecific crossability in blue and Engelmann spruce; (2) determine the inheritance of isozymes in megagametophyte, embryo and bud tissues in blue and Engelmann spruce; (3) describe and quantify the isozyme variability in blue and Engelmann spruce in the Dolores River drainage in southwestern Colorado; (4) determine if isozyme analysis can be used to identify blue-Engelmann spruce hybrids; and (5) determine if natural hybrids between blue and Engelmann spruce exist in the Dolores River drainage and look for evidence of introgression. This work is part of a long-range study investigating the genetic basis of adaptation in the North American spruce complex (Hanover 1975).

CHAPTER II

GENETIC VARIATION AND CONTROL OF INTRASPECIFIC CROSSABILITY IN BLUE AND ENGELMANN SPRUCE

ABSTRACT

To assess the degree of genetic control for intraspecific crossability in blue and Engelmann spruce, two 20-parent partial diallel matings, one for each species, were conducted in the field during the spring of 1983. As measures of crossability, percent germinated and percent ungerminated-but-full seed on a total seed basis--full and empty--were determined for the individual crosses. Best linear unbiased prediction (BLUP) was used to estimate the individual fixed and random effects of the mixed model, and restricted maximum likelihood (REML) methods were used to estimate general combining ability (GCA), specific combining ability (SCA), maternal effects and error variances.

Engelmann spruce exhibited slightly higher intraspecific crossability than was observed for blue spruce. Viable seed yields from selfed crosses were approximately 50 percent less than control-pollinated biparental seed yields. Nonadditive sources of variation apparently exert major genetic influence in the production of viable seed in both species. Maternal effects are very important in the production of viable seed in blue spruce,

but not so in Engelmann spruce. Therefore, to maximize production of viable seed in seed orchards of both species, parents should be selected on the basis of specific cross combinations which produce proportionately high amounts of viable seed. The practical effect of maternal influence in blue spruce seed production should be studied further.

The use of best linear unbiased prediction techniques should have wide utility in plant breeding because of their ready application to unbalanced multifactor data.

INTRODUCTION

Phenotypic improvement of a trait requires an understanding of the genetic crontrol governing the character of interest and how the environment influences its expression. Only then can appropriate selection and breeding methods be applied to the population or individuals to bring about the desired gains. In forest trees, as in all commercially important plant species, there are a wide variety of traits for which improvement is desired. In conjunction with these efforts, the improved plant material must be propagated either sexually--via seed--or asexually--via vegetative propagation--in large numbers for commercial use. Therefore an equally important trait is how well do the individuals selected on the basis

of yield or some other trait propagate and what is its genetic control.

Many of the commercially important characters in forest trees are under polygenic control (Wright 1976). Therefore long-term gains are best realized through breeding programs and mass selection techniques which utililze the additive genetic variance associated with these traits (Hallauer 1981). Because of this, most forest trees in North America are propagated by seed rather than vegetatively. Also, vegetative propagation is not yet feasible on a commercial scale for many important forest tree species, especially among conifers (Timmis and Ritchie 1984; Bonga 1981). In addition, because it takes so long for many tree species to reach commercial maturity (from 20 to 200 years), a variety of genotypes is generally more desirable than a monoculture (Tigerstedt 1974).

The objectives of this study were to (1) assess the genetic variation and control of intraspecific crossability in blue and Engelmann spruce, and (2) generate full-sib progeny for future studies on the inheritance of a wide variety of traits in both species. The information on intraspecific crossability is desirable for two reasons. It will serve as a reference for studying the interspecific crossability between blue and Engelmann spruce (see Ernst et al. 1985c). Also, it will provide information important to seed orchard selection and management techniques for the two species. Blue spruce is one of the most widely planted

horticultural conifers in the world (Hanover 1975), and Engelmann spruce is a commercially valuable timber species in the Rocky Mountain region of North America (Fowler and Roche 1975).

The Dolores River drainage in southwestern Colorado was selected as the study site because both blue and Engelmann spruce occur in the drainage, often in close proximity. Also, previous studies investigating the genetic variability in morphological and terpenoid characters of blue and Engelmann spruce have been conducted in the drainage (Hanover 1975; Reed and Hanover 1983; Schaefer and Hanover 1985a and b).

The partial diallel mating design is theoretically efficient in regards to the amount of information per cross and accuracy of the estimates of the genetic parameters (Namkoong and Roberds 1974; Pederson 1972; Gordon 1980). Empirical evidence has also shown the partial diallel to be generally efficient (Chaudharey et al. 1977; Anand and Murty 1969; Murty et al. 1967; Kearsey 1965).

Best linear unbiased prediction (BLUP), as developed by Henderson (1973) and described by Mao (1982), can be viewed in practice as an extension of generalized least-squares (GLS)--or more specifically, best linear unbiased estimation (BLUE)--techniques which allow for simultaneous estimation of fixed effects and prediction of random effects. While BLUE is applicable only to fixed model

analysis, BLUP was developed for analysis of mixed models which incorporate a mixture of random and fixed factors. In BLUP, a priori variances and covariances--from previous studies or estimates--associated with the different random factors are incorporated into the mixed model equations (MME) used to obtain the solutions. Using a maximum likelihood or restricted maximum likelihood (REML) technique such as described by Schaeffer (1976), new variance and covariance estimates can be obtained from the BLUP solutions, and the process is repeated until convergence. BLUP is especially well suited to unbalanced data situations, which are very prevalent in plant breeding experiments.

MATERIALS AND METHODS

The Dolores River was divided elevationally into five species-occupation zones, the middle three zones (2,3 and 4) comprising the sample area for this study. Zone 2 extends from 2400 to 2590 meters (m) in elevation and blue spruce is the primary occupant relative to the occurrence of Engelmann spruce. Zone 3 extends from 2590 to 2770 m, an elevationally intermediate zone with respect to the habitats of blue and Engelmann spruce, both species occurring in zone 3 and often in close proximity. Zone 4 extends from 2770 to 2960 m and is occupied primarily by

Engelmann spruce relative to the presence of blue spruce. The respective parents in zones 2 and 4 represent "pure" species subpopulations and parents in zone 3 represent putative introgressed subpopulations. All parents were readily identifiable as to species. During the spring of 1983, ten blue spruce trees each in zones 2 and 3 and ten Engelmann spruce trees each in zones 3 and 4--for a total of 40 trees, 20 of each species--were selected primarily on the basis of fecundity, accessability and climbability to be used as parents in the controlled pollinations.

The partial diallel design used in this study incorporated three intraspecific crosses--including selfs--and three interspecific crosses per parent (Figure 1). During the spring of 1983, female strobili were isolated before pollen shed. Also, pollen strobili were collected just prior to anthesis, dried and the pollen extracted. Standard pollination methods for conifers were utilized and the trees were debagged following scale closure of the female strobili. The female strobili on a few of the blue spruce parents were beginning to close when pollinated, and therefore possible receptivity differences based on scale closure were recorded. Female strobili with scales completely open and fully receptive were scored as 1, strobili with up to 50 percent of the scales beginning to close were scored as 2, and strobili with more than 50 percent of the scales beginning to close scored as 3. The

Female \ Male	1	2	3	4	5	6	7	8	9	10	11
1	X					X					X
2		X					X				
3			X					X			
4				X					X		
5					X					X	
6	X					X					X
7		X					X				
8			X					X			
9				X					X		
10					X					X	
11	X					X					X

Figure 1. A portion of the partial diallel mating design layout used to make the controlled pollinations. An "X" denotes an attempted full-sib cross.

ages of the respective parents were also determined using increment cores taken from the bole at a height of one meter.

In the fall of 1983, control and open-pollinated cones were collected. The control-pollinated cones were kept separate by isolation bag. The cones were dried and seed extracted by hand, recording the total number of cones per bag and the number of cones damaged by insects per isolation bag. The extracted seed was blown to separate empty and putatively full seed and both portions were counted and kept in cold storage (4° C).

During the summer of 1984, germination tests were conducted using a maximum--depending on availability--of 30 seeds per isolation bag. Blue and Engelmann spruce seed do not have any special dormancy requirements (Heit 1961). The seeds were placed in 6 cm diameter petri dishes, using two filter paper discs (Whatman, No. 3) as the germination medium. A solution of 2.5g/l Captan and Benlate was used to keep the seeds moist and deter fungal growth during germination. The seeds were germinated in partial light at a day-night temperature cycle of 27 and 18° C, respectively. The seeds were observed daily for 30 days, and the number of newly germinated seeds recorded each day. Upon germination, the seeds were removed and planted in 5 x 5 x 25 cm plant bands containing 3:1:1 (peat:vermiculite:perlite) soil mix. At the end of the 30-day germination period, the number of ungerminated seeds

were counted and then dissected to determine the number of full but ungerminated seed and empty seed. The percentages of germinated and ungerminated-but-full seed were determined from the germination test (subsample), and these values were then extrapolated to a total seed basis--the total number of full and empty seed per isolation bag--to serve as the dependent variables in the analysis. Percent germinated seed on a total seed basis was used as a measure of intraspecific crossability because it measures the number of viable seed produced for a given cross. Percent ungerminated-but-full seed was measured primarily to detect postzygotic abnormalities.

The model equation used to account for the identified fixed and random factors was:

$$Y_{ijklmn} = c + L_i L_k + F_{ijm} + M_{kl} + (F_{im}M_k)_{jl} + R_m + b_a A + b_c C + b_d D + e_{ijklmn}$$

where:

Y_{ijklmn} = the nth germination record (percent germinated or percent ungerminated-but-full seed on a total seed basis) of the cross between the jth female, found at location i and of receptivity class m and of age A, with male l of location k, that record (bag) having C cones and D (percent) of those cones damaged by insects;

c = a constant common to all parents;

F_{ijm} = the random effect of female j which resides in

location i and of receptivity class m (j = 1,...,20);

M_{kl} = the random effect of male l which resides in location k (l = 1,...,20);

$(F_{im}M_k)_{jl}$ = the random interaction effect common to all records (bags) of the subclass corresponding to the cross of female j (from location i and of receptivity class m) and male l (from location k);

L_iL_k = a subclass effect combining the fixed effects of the location of female j, the location of male l, and the location interaction (i,k = 1,2 or 3 for zones 2, 3 and 4, respectively);

R_m = the fixed effect of the receptivity class of female j (m = 1,2 or 3, as described previously);

A = the age of female j in years;

b_a = the regression coefficient corresponding to the age of female j;

C = the number of cones in isolation bag n of cross $(F_{im}M_k)_{jl}$;

b_c = the regression coefficient corresponding to the number of cones in record (bag) n of cross $(F_{im}M_k)_{jl}$;

D = the percentage of cones in isolation bag n of cross $(F_{im}M_k)_{jl}$ that were damaged by insects;

b_d = the regression coefficient corresponding to the percent of insect-damaged cones in record (bag) n of cross $(F_{im}M_k)_{jl}$;

e_{ijklmn} = the random residual (error) associated with record y_{ijklmn}.

The model equation is rewritten in matrix form to facilitate the computation:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Zu} + \mathbf{e}$$

where:

y = an a x 1 vector of the germination records, where a = 91 for blue spruce and 96 for Engelmann spruce intraspecific crosses;

b = an unknown b x 1 vector containing the different levels of the fixed factors and the regression coefficients as described previously, where b = 11 for blue spruce and 8 for Engelmann spruce crosses;

X = a known a x b matrix, its elements denoting the levels of the fixed factors and the values of the covariates in the **b** vector which are associated with a given **y** observation (record);

u = an unknown c x 1 vector containing the different levels of the random factors--female, male and female x male effects--where c = 98 for blue spruce and 100 for Engelmann spruce crosses;

Z = a known a x c matrix of ones and zeros, its elements denoting the levels of the random factors in the **u** vector which are associated with a given **y** observation (record);

e = an unknown a x 1 vector of random residuals.

The two groups of intraspecific crosses were analyzed separately.

Assumptions pertaining to the operational model were: (1) the parents were unrelated; (2) the parents of each species were from a single, randomly mating population (and therefore share common female, male and female x male variances for each trait); and (3) the control pollinations were made at random (no selection). Based on these assumptions, the mathematical expectations of the variances were:

$$\text{Var} \begin{bmatrix} \mathbf{y} \\ \mathbf{u} \\ \mathbf{e} \end{bmatrix} = \begin{bmatrix} \mathbf{V} & \mathbf{ZG} & \mathbf{R} \\ \mathbf{GZ'} & \mathbf{G} & \mathbf{0} \\ \mathbf{R} & \mathbf{0} & \mathbf{R} \end{bmatrix}$$

where:

$\mathbf{G} = \mathbf{I}\sigma_i^2$, where **I** is a c x c identity matrix, and $\sigma_i^2 = \sigma_F^2$, σ_M^2 or σ_{FM}^2, corresponding to variances associated with the female, male and female x male random effects, respectively;

$\mathbf{R} = \mathbf{I}\sigma^2$, where **I** is an a x a identity matrix and σ^2 the error variance associated with the random residuals;

and $\mathbf{V} = \mathbf{ZGZ'} + \mathbf{R}$.

The fixed effects and covariates were considered to be "nuisance" factors because they were included in the model only to improve the accuracy of the variance estimates associated with the random factors. Estimation of the variances was the primary focus of this study.

To solve a set of mixed model equations (MME), restrictions must be imposed in order to obtain solutions for the fixed effects. For this model, the female, male and female x male (random) effects have unique solutions, but the fixed effects are not individually estimable (unique). However, certain linear contrasts between the levels of the fixed effects are best (minimum variance of the estimator), linear, unbiased and estimable or unique (BLUE).

The solutions for the random factors in mixed model prediction (BLUP) are unique. However, nested predictands are the sum of fixed and random effects in mixed model prediction--e.g., location i + female j, and location k + male l--and are not unique. Therefore, direct comparisons of the female, male and female x male predictands are possible only within a common location or receptivity class. If the effects of the nested fixed factors are accounted for (i.e., adjusted in linear combination), comparisons of parents or crosses from different locations or classes are estimable or unique.

The female, male and female x male predictands correspond to general and specific combining ability estimates of the respective parents and crosses in the partial diallel mating design. Under the assumptions that the population was sampled at random, it is randomly mating, and there is no inbreeding, linkage or epistasis,

the general combining ability variance (σ_G^2) corresponds to one-fourth the total additive genetic variance ($1/4\ \sigma_A^2$) for that trait, and the specific combining ability variance (σ_S^2) corresponds to one-fourth the total nonadditive (dominance) variance ($1/4\sigma_D^2$) in the partial diallel mating design (Kempthorne and Curnow 1961). Maternal effects were contained within the female general combining ability estimate, and therefore the maternal effect variance (σ_{ME}^2) is estimable by subtracting the male general combining ability variance from the female general combining ability variance: $\sigma_{ME}^2 = \sigma_{GF}^2 - \sigma_{GM}^2$.

Variance component estimates were obtained using iterative expectation maximization restricted maximum likelihood (EM-REML) algorithms (Banks et al. 1985) under the assumption of normality of female, male and female x male effects. The EM-REML algorithms used were:

$$\hat{\sigma}^2 = (y'y - \hat{b}'X'y - \hat{u}'Z'y)/n - 1$$

$$\hat{\sigma}_i^2 = (\hat{u}'\hat{u} - \hat{\sigma}^2 tr(C))/p_i - 1$$

where $\hat{b}$ and $\hat{u}$ were the solutions to the MME. The vector $\hat{u}_i$ corresponds to the female, male or female x male portion of $\hat{u}$ used to estimate the respective variances. The C matrix is the portion of the inverse matrix from the MME pertaining to female, male or female x male effects. The value n is the total number of records, and p_i is the number of parents or crosses associated with the female, male or female x male effects in each set of intraspecific crosses. The variance estimates were incorporated into the

MME, and new solutions for **b** and **u** and variance estimates were obtained. Iterations were carried out until the estimated variances changed less than one percent from the previous iteration.

RESULTS

Viable seeds were obtained from 46 blue spruce and 56 Engelmann spruce full-sib families, out of a total 60 possible full-sib families for each species. Mean values of the percent germinated and percent ungerminated-but-full seed from open-pollinated and control-pollinated selfed and biparental collections are given in Table 1. Viable seed yields for open-pollinated crosses exceeded those for control-pollinated biparental crosses by a factor of two or more. Larger yields for open-pollinated collections were expected because only one pollen parent was used in each pollination bag, whereas there is a diverse mixture of pollen genotypes under open-pollinated conditions. Viable seed yields for selfed crosses in both species were 80 to 90 percent less than those for open-pollinated accessions, and approximately 50 percent less than control-pollinated biparental cross yields. Slightly higher intraspecific crossability was observed for Engelmann spruce than for blue spruce among both open and control-pollinated collections (Table 1).

The percent ungerminated-but-full seed yields, when compared to percent germinated values, were proportionately consistent across all pollination types for both species (Table 1). There was only a slight proportionate increase in full-but-ungerminated seed among the selfed crosses relative to open-pollinated and biparental crosses.

All variance component estimates converged rapidly using restricted maximum likelihood techniques. Due to the small magnitude of the ungerminated-but-full male general combining ability variance in Engelmann spruce, iterations for this variance component were terminated after a change of less than five percent rather than one percent. The variance component and narrow-sense heritability estimates are given in Table 2.

Among the blue spruce crosses, general combining ability (GCA) variance accounted for only one percent of the total variance observed in percent germination (Table 2). Maternal effects and specific combining ability (SCA) variances greatly exceeded the GCA variance for this trait, accounting for 15 and 56 percent of the total variance, respectively. For percent germination in Engelmann spruce, GCA varaince was also much smaller than SCA variance, each accounting for one and 47 percent of the total variance in this trait, respectively (Table 2). The most striking difference observed between the species in regards to the production of viable seed was that the maternal effects

Table 1. Mean values of percent germinated (% Germ) and percent ungerminated-but-full (% Ungf) seed on a total seed basis for open-pollinated (Open) and control-pollinated (selfed and biparental) collections.

Species		Open		Self		Biparental	
		%Germ	%Ungf	%Germ	%Ungf	%Germ	%Ungf
Blue spruce	Mean	43.1	4.9	4.8	1.0	11.9	2.2
Engel. spruce	Mean	48.7	8.0	9.5	2.3	19.7	3.6

Table 2. Variance component and narrow-sense heritability estimates for production of viable and abnormal full-sib seed in blue and Engelmann spruce. Numbers in parentheses represent percentages of the respective variance components as compared to the total observed variance for that trait.

Trait[a]	Species[b]	Variance Component				h^2
		GCA	SCA	Maternal	Error	
%Germ	BS	1.890	30.992	118.482	57.796	0.04
		(1)	(15)	(56)	(28)	
	ES	3.987	135.299	-0.025	150.086	0.06
		(1)	(47)	-----[c]	(52)	
%Ungf	BS	2.642	1.028	-0.357	5.711	1.12
		(28)	(11)	-----[c]	(61)	
	ES	0.687	2.712	2.834	15.000	0.12
		(3)	(13)	(13)	(71)	

[a] %Germ = percent germinated, %Ungf = percent ungerminated-but-full seed on a total seed basis.
[b] BS = blue spruce, ES = Engelmann spruce.
[c] Not calculated due to small, negative variance components (assumed to be zero).

variance was essentially zero in Engelmann spruce. These results suggest maternal influences are very important in the production of viable seed in blue spruce but are not in Engelmann spruce. In both species, sources of nonadditive (dominance) variance apparently exert a great deal more control over viable seed yields than do additive sources of genetic variation, and this is reflected in the very small narrow-sense heritability estimates for this trait in both blue and Engelmann spruce (Table 2).

GCA and SCA variances were both relatively large for percent ungerminated-but-full seed yields in blue spruce, accounting for 28 and 11 percent, respectively, of the total variance observed in this trait (Table 2). However, GCA variance was disproportionately large, resulting in a narrow-sense heritability estimate exceeding unity. Maternal effects variance was essentially zero for this trait in blue spruce. In Engelmann spruce, GCA variance was relatively small, accounting for only three percent of the total variance. However, both SCA and maternal effects variance were moderately large and approximately equivalent in magnitude for percent ungerminated-but-full seed yields in Engelmann spruce, each accounting for 13 percent of the total observed variance (Table 2). In both species, the number of full but nonviable seed may be under the control of nonadditive sources of variation as well. Only for Engelmann spruce was there any evidence of maternal influences in the production of ungerminated-but-full seed.

Product-moment correlations between the two germination traits, calculated using parental means or GCA estimates, were somewhat contrasting for the two species (Table 3). Blue spruce parents--serving as either female or male--which produced higher viable seed yields also produced higher ungerminated-but-full seed yields. However, for Engelmann spruce there was no correlation between the two germination traits, or possibly a slight negative association when Engelmann spruce parents served as males.

Product-moment correlations between male and female mean responses for percent germination in both species were calculated. Both blue and Engelmann spruce indicated a slight degree of positive association between male and female responses on a parental mean basis--$r = 0.28$ for blue spruce and 0.23 for Engelmann spruce.

DISCUSSION

In both blue and Engelmann spruce, the production of viable seed is primarily under the control of nonadditive (dominance) sources of genetic variation. A large amount of the total variance observed in both species for percent germination on a total seed basis was attributable to SCA variance, while very little was accounted for by GCA variance. Therefore, very little gain will be achieved in

Table 3. Product-moment correlations between percent germinated and percent ungerminated-but-full values when parents served as females or males, derived using parental means and general combining ability evaluations (BS = blue spruce, ES = Engelmann spruce).

	Females		Males	
	BS	ES	BS	ES
Parental means	0.46	-0.01	0.24	-0.15
GCA estimates	0.40	-0.30	0.44	-0.33

the production of viable intraspecific seed in blue and Engelmann spruce by using half-sib selection techniques. Rather, selection must be based on specific cross combinations which show high crossability rates as measured by germination tests.

Morgenstern (1974) observed in a diallel cross of seven black spruce (Picea mariana (Mill.) B.S.P.) parents that SCA variance accounted for six times the total variance observed for percent germination than did GCA variance. Morgenstern attributed this to the fact that percent germination and percent survival, for which similar results were obtained, are fitness or survival traits, which are less likely to be controlled by additive sources of variation (Falconer 1960). Morgenstern (1974) observed in the same study that for traits related to growth and size--rate of germination, first and second-year height--additive sources of variation were of primary importance. In a six parent diallel cross of flax (Linum usitatissimum L.), percent germination was also controlled primarily by genes showing dominance (Gupta and Basak 1983). Both additive and nonadditive sources of variation were important in low-temperature germination percentages in cucumber (Cucumis sativus L.) (Wehner 1984). Additive sources of variation were more important than nonadditive sources in seed yield of tall fescue (Festuca arundinacea Schreb.) (Nguyen and Sleper 1983). Therefore, while

nonadditive sources of variation do not exert major control in the production of viable seed among all plant species, results similar to those observed in this study have been reported for a variety of other plant species.

Maternal influences were very important in the production of viable seed in blue spruce, but of no apparent importance in Engelmann spruce. Large maternal influences in seed germination have also been observed in black spruce (Morgenstern 1974), Virginia pine (Pinus virginiana Mill.) (Bramlett et al. 1983), and Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) (Greathouse 1966). Falconer (1960) has interpreted maternal effects as common environmental influences of a given female parent. Bramlett et al. (1983) have further subdivided maternal effects for members of the Pinaceae family into the effect of the local environment of the female parent and the effect of the seedcoat and megagametophyte tissue--both maternally derived--of the germinating seed. The megagametophyte seed tissue in members of Pinaceae is haploid and identical in genetic composition to the female egg nuclei. Therefore the only paternal influence in the developing seed is through the embryo. Whether the observed difference between blue and Engelmann spruce in maternal influence of seed set is due only to how each species responds to the local environment or species-specific responses to seed coat and megagametophyte effects is uncertain. As Bramlett et al. (1983) suggested, the

only way to test the relative importance of the two effects would be to replicate given crosses in clonally derived seed orchards located on a variety of sites. The effect of local environment only on the production of viable seed could be assessed by replicating given crosses in separate seedling seed orchards located on two or more sites, each orchard made up of the same half-sib or full-sib families. The magnitude of maternal effects should be investigated further for seed orchards of blue spruce.

The variance of female parental means for percent germination in blue spruce was 75 percent greater than that observed among male responses. In Engelmann spruce the variance of female means exceeded that observed among males by 37 percent. The large maternal influences observed in blue spruce may account for some of the increased variance observed among females of this species.

Selfing reduced viable seed yields approximately 50 percent relative to biparental controlled crosses in both blue and Engelmann spruce. This is consistent with other reports on the effect of selfing in blue spruce (Cram 1984). Conifers are generally intolerant of inbreeding, resulting in reduced levels of seed set and poor survival and growth of selfed seedlings (Franklin 1970; Shaw and Allard 1981). While selfed crosses in both species did have lower yields of viable seed, they did not produce proportionately more ungerminated-but-full seed relative to

the proportions observed among biparental controlled crosses. Therefore reductions in seed set due to selfing in blue and Engelmann spruce were probably the result of prezygotic incompatibility mechanisms that prevented normal fertilization, as has been reported for other conifers (Fechner 1979). Some loss of seed may occur between fertilization and early embryo development (Cecich 1979; Kossuth and Fechner 1973; Allen and Owens 1972). Both prezygotic incompatibility and postzygotic inviability have been observed among members of the Pinaceae family (Fechner 1979).

For some as yet unknown reason, there was a slight tendency for blue spruce parents which produced more viable seed on average to also produce greater numbers of nonviable full seed. There was no such trend--or possibly a slight reversal--observed among Engelmann spruce parents. This species difference is apparently not due to the maternal effects observed to influence seed set in blue spruce, as the trend was consistent in both species when the parent served as either male or female. Also, it will not account for the slightly higher intraspecific crossability observed for Engelmann spruce, as the proportion of ungerminated-but-full seed relative to viable seed was the same in both species for the respective pollination types.

CHAPTER III

INHERITANCE OF ISOZYMES IN SEED AND BUD TISSUES OF BLUE AND ENGELMANN SPRUCE

ABSTRACT

Thirteen loci from 11 enzyme systems were identified among full-sib and half-sib progeny of blue and Engelmann spruce. Eleven of the loci were expressed in bud, embryo and megagametophyte tissue; the reamaining two loci were expressed only in embryo and megagametophyte tissue. There were no mobility differences observed between loci expressed in seed and bud tissues.

The mode of inheritance for ten of the loci was confirmed based on progeny genotypic distributions. For the two loci not expressed in bud tissue, acid phosphatase(2) (ACP(2)) and diaphorase(2) (DIA(2)), inheritance was inferred from pooled segregation ratios of megagametophytes from open-pollinated half-sib seed from heterozygous females. The inheritance of glutamate oxaloacetate transaminase(3) (GOT(3)) was also inferred from segregation ratios and diploid embryo phenotypes of open-pollinated progeny due to a lack of variability at this locus among the 40 parents in the mating design. Two loci, aldolase (ALD) and malate dehydrogenase (MDH(2)), were monomorphic among the 20 parents of both species.

INTRODUCTION

Isoenzyme analysis is a useful tool in assessing the population genetic structure of a species and its evolutionary relatedness to other species. Allelic frequencies can be determined for a large number of loci which have various--and generally unknown--degrees of selective neutrality: e.g., 71 loci sampled in a study of humans in Europe (Harris and Hopkinson 1972); 42 loci sampled in a study of lodgepole pine (Pinus contorta Dougl.) in western North America (Wheeler and Guries 1982). Gene flow, drift and mutations among subpopulations of a species or between species can be quantified (e.g., Millar 1983; Shaw and Allard 1981; Muller 1977; Mitton et al. 1977; Adams and Coutinho 1977). However, the mode of isozyme inheritance must be known before one can accurately estimate and interpret measures of variation, gene flow, fitness, mutation or genetic relatedness.

The question of whether blue and Engelmann spruce (Picea pungens Engelm. and P. engelmannii Parry ex Engelm., respectively) hybridize naturally has been studied extensively but no clear conclusions have been drawn. Morphological and biochemical data have proven relatively effective in distinguishing the two species and trees of intermediate phenotypes have been identified (Daubenmire 1972; Taylor et al. 1975; Mitton and Andalora 1981;

Schaefer and Hanover 1985a and b). Isozymes may also prove useful in distinguishing hybrids between blue and Engelmann spruce and subsequent introgression because they represent more closely the variability at the DNA level, but first the inheritance patterns of the isozymes must be determined. The objective of this study was to determine the inheritance patterns of isozymes in megagametophyte, embryo and bud tissues in blue and Engelmann spruce.

The Dolores River drainage in southwestern Colorado was selected as the study site because both blue and Engelmann spruce occur in the drainage, often in close proximity. Also, previous studies investigating the genetic variabililty in morphological and terpenoid characters of blue and Engelmann spruce have been conducted in the drainage (Hanover 1975; Reed and Hanover 1983; Schaefer and Hanover 1985a and b).

MATERIALS AND METHODS

The Dolores River was subjectively divided into five elevational species-occupation zones. The parents used to generate the full-sib progeny in this study were located in the middle three zones (2,3 and 4). Zone 2 represents an essentially pure blue spruce zone relative to the occurrence of Engelmann spruce and extends from 2400 to 2590 meters (m) in elevation. Zone 3 is an elevationally

intermediate zone relative to the habitats of blue and Engelmann spruce, extending from 2590 to 2770 m, with both species present in this zone and often in close proximity. Zone 4 is a predominately Engelmann spruce zone and extends from 2770 to 2960 m in elevation. Twenty trees of each species--ten blue spruce trees each in zones 2 and 3, and ten Engelmann spruce trees each in zones 3 and 4--were selected to serve as parents for the controlled pollinations. Selection was based primarily on fecundity, accessability and climbability. All parents were readily identifiable as to species.

A partial diallel mating design was utilized to carry out the pollinations in the field during the spring of 1983. Both intra- and interspecific crosses were made. Only the intraspecific progeny will be evaluated in this paper, and the results from the interspecific crosses will be reported elsewhere (Ernst et al. 1985c). For a more detailed description of the partial diallel mating design used in this study, see Ernst et al. (1985). Three intraspecific crosses were made per female, including one self and two biparental crosses. The biparental crosses were reciprocated on the respective females.

In the fall of 1983, control-pollinated and open-pollinated cones were collected from each parent. The cones were dried and seed extracted by hand. In the fall of 1984, dormant vegetative buds were collected from each

parent tree and stored at -20°C until used in the electrophoretic analysis.

The extracted seed from the controlled crosses were blown to remove empty seeds. Germination tests were conducted with the blown seed during the summer of 1984 (see Ernst et al. (1985) for results of the germination tests and measures of crossability for the intraspecific crosses), and then each seedling was planted in a 5 x 5 x 25 cm plant band containing 3:1:1 (peat:vermiculite:perlite) soil mix. The seedlings were grown under accelerated-optimal-growth conditions (Hanover et al. 1976) from August, 1984, until January, 1985, when the seedlings were allowed to go dormant. Dormant vegetative buds were collected from the progeny seedlings in March, 1985, and stored at -20°C until used in the electrophoretic analysis.

Parental genotypes were determined by simultaneous comparison of isozymes in bud, embryo and gametophyte tissues. After partial germination--radicle protruding more than 3 mm--and removal of the seed coat, the megagametophyte and embryo from ten open-pollinated seeds from each female were separated and each placed into a 0.5 ml polystyrene sample vial. Apical domes from five dormant vegetative buds from the same female tree were placed into separate sample vials. The sample vials were then put in foam storage blocks, wrapped in plastic wrap and frozen at -20°C until the day they were used.

Because of the relatively small number of seed available from each cross (family), only bud tissue from the seedlings was used to characterize full-sib progeny genotypes. They were determined using one dormant vegetative bud from each full-sib seedling, with a maximum of ten seedlings per family analyzed. Each bud was placed in a 0.5 ml sample vial and stored at -20°C until used.

To determine the inheritance of loci not expressed in bud tissue (i.e., expressed only in megagametophyte and/or embryo tissue), isozymes were assayed using megagametophytes from seed of open-pollinated half-sib collections of blue and Engelmann spruce. These collections were made in the Dolores River drainage, primarily for use in another study (Ernst et al. 1985b). Eight megagametophytes were prepared as previously described and used in electrophoresis.

Table 1 gives the composition of the electrophoresis buffers, power requirements, and references for each. Table 2 lists the enzymes analyzed in each buffer system and references for the staining recipes. Connaught starch, lot 400-1 (Connaught Laboratories, Willowdale, Ontario, Canada), was utilized throughout the study to prepare the 12.5 percent w/v starch gels. The thickness of the gel varied with the buffer system used: 20 mm gels for buffer system I and 16 mm gels for buffer system II. The electrophoresis equipment and techniques were similar to those described elsewhere (O'Malley et al. 1980).

In preparation for electrophoresis, the sample vials containing bud, megagametophyte and embryo tissues were removed from the freezer and placed in a plexiglass block embedded in ice. Two or more drops of extraction buffer (Wendel and Parks 1982) were placed in each vial and the tissues homogenized using a motor-driven teflon grinding tip. The homogenate was absorbed onto 2 x 20 mm filter paper wicks (Whatman, No. 3) and inserted into the vertical slice at the gel origin.

Electrophoresis was conducted in a forced-air refrigerator at 4°C, maintaining the amperage settings as given in Table 1. Wicks were removed after 15 minutes, and then electrophoresis was continued for 5.0 and 4.5 hours, respectively, for buffer systems I and II. These run durations ensured a front migration of 8 cm, as determined by bromophenol blue dye markers. The gels were then sliced horizontally, immersed in staining solution and incubated at 37°C in the dark until resolved. The stain recipes used were similar to those referenced in Table 2. All enzyme systems assayed in this study migrated anodally--no isozymes appeared in stained cathodal gel slices.

For enzyme systems controlled by more than one locus, the fastest migrating zone was designated as locus 1, the next fastest 2, etc.. Multiple allozymes within each locus were numbered in the same manner; the most anodal was labeled as allele 1, etc.. Mobilities of the different isozymes were quantified relative to the buffer front (R_f).

Table 1. Electrophoresis buffers and respective power requirements.

Electrophoresis Buffer System			
No.	Electrode	Gel	Power
I[a]	0.125 M Tris pH 7.0 with 1.0 M citric acid	0.05 M DL-histidine-HCl 1.40 mM EDTA ph 7.0 with 1.0 M Tris Dilute 5:1 (4 water: 1 stock)	50 ma
II[b]	0.029 M lithium hydroxide 0.19 M boric acid pH 8.3 with 10 N NaOH	10% electrode buffer 0.05 M Tris 0.0076 M citric acid pH 8.3 with 10 N NaOH Dilute 10:1 (9 water: 1 stock)	75 ma

[a] Cheliak and Pitel 1984.
[b] Scandalios 1969.

Table 2. Enzyme systems assayed in respective buffer systems.

Buffer System	Enzyme	E.C. No.	Reference[a]
I	ACO	4.2.1.3	1
	ACP	3.1.3.2	1
	ALD	4.1.2.13	2
	G6P	1.1.1.49	2
	IDH	1.1.1.42	1
	MDH	1.1.1.37	1
	6PG	1.1.1.44	2
	SKDH		2
II	DIA	1.6.4.3	1
	GDH	1.4.1.3	1
	GOT	2.6.1.1	2
	PGI	5.3.1.9	2
	PGM	2.7.5.1	2

[a] 1 = O'Malley et al. (1980); 2 = Conkle et al. (1982).

The modes of inheritance of isozymes expressed only in bud tissue were evaluated by comparing the observed distribution of progeny genotypes to the expected distribution--based on parental genotype designations--using the log-linear G-statistic (Sokal and Rohlf 1969). For polymorphic loci which were expressed only in megagametophyte--and sometimes embryo--tissue, inheritance was determined by comparing pooled segregation ratios of megagametophytes from half-sib open-pollinated families of heterozygous mother trees using the same log-linear G-statistic. The probability of misclassifying a heterozygote at a given locus is $(1/2)^{n-1}$, where n equals the number of megagametophytes analyzed per family. In this study, n = 8, and therefore the probability of misclassifying a heterozygote was $(1/2)^7 = 0.0078$.

RESULTS AND DISCUSSION

From a total 60 possible intraspecific full-sib families for each species group, viable progeny were obtained for 49 blue spruce and 56 Engelmann spruce families. Of the 13 enzyme systems assayed (Table 2), 11 produced sufficiently clear band patterns using seed or bud tissues to make putative locus and allele assignments; only glucose-6-phosphate dehydrogenase (G6P) and shikimic

dehydrogenase (SKDH) did not. G6P was weakly staining in all blue spruce tissues assayed, while in Engelmann spruce only megagametophyte tissue produced clear band patterns. The band patterns of G6P in blue and Engelamnn spruce resembled those observed in ponderosa pine (Pinus ponderosa Dougl. ex Laws.) (O'Malley et al. 1979). SKDH produced clear bands in all tissues, but no genetic interpretation could be discerned. Another buffer system for this enzyme may help achieve a genetically explainable separation of electromorphs (e.g., see Conkle et al. 1982).

Aconitase

One zone of activity was observed on gels stained for ACO, with variation in this zone occurring in both blue and Engelmann spruce. Bands were best resolved in megagametophyte tissue, but also resolved well in embryo and bud tissue. The same two electromorphs were observed in both species, and they are inherited as expected for a one locus system (Table 3). Heterozygous diploid tissue expressed a three-banded phenotype (Figure 1), indicating the functional enzyme may be a dimer. The functional form of ACO in humans is monomeric (Slaughter et al. 1975; Zouros 1976), but its subunit structure has not been determined in conifers (El-Kassaby et al. 1982; Guries and Ledig 1978).

Acid phosphatase

Two zones of activity were observed on gels stained for ACP. Only the slower of the two zones, ACP(2),

Table 3. Single-locus segregation tests of genotypes expressed in bud tissue of blue and Engelmann spruce full-sib progeny.

Enzyme (locus)	Species[a]	Type of Cross[b]	No. Crosses	No. Progeny	Progeny genotypes--observed(expected) 1/1	2/2	3/3	1/2	1/3	2/3	3/4	G(df)[c]
ACO	BS	1/1 x 1/2	10	75	38(37.5)			37(37.5)				0.01(1)
		1/2 x 1/2	8	51	10(12.75)	16(12.75)		25(25.5)				1.42(2)
		1/2 x 2/2	9	73		40(36.5)		33(36.5)				0.67(1)
	ES	1/1 x 1/2	6	59	34(29.5)			25(29.5)				1.38(1)
		1/2 x 1/2	9	56	14(14.0)	13(14.0)		29(28.0)				0.11(2)
		1/2 x 2/2	13	122		68(61.0)		54(61.0)				1.61(1)
IDH(2)	ES	1/1 x 1/2	1	10	3(5.0)			3(5.0)				1.65(1)
		1/2 x 1/2	2	11	4(2.75)	1(2.75)		6(5.5)				2.02(2)
		1/2 x 2/2	10	94		54(47.0)		40(47.0)				2.09(1)
6PG(1)	BS	1/1 x 1/3	12	108	59(54.0)				49(54.0)			0.93(1)
		1/3 x 1/3	6	33	7(8.25)		10(8.25)		16(16.5)			0.56(2)
	ES	1/1 x 1/2	20	190	105(95.0)			85(95.0)				2.11(1)
		1/2 x 1/2	7	38	11(9.5)	9(9.5)		18(19.0)				0.31(2)
GDH	ES	1/1 x 1/2	22	213	99(106.5)			114(106.5)				1.06(1)
		1/2 x 1/2	8	35	7(8.75)	9(8.75)		19(17.5)				0.51(2)
		1/2 x 2/2	6	60		21(30.0)		39(30.0)				5.48(1)
PGI(2)	BS	2/3 x 3/3	3	29			15(14.5)			14(14.5)		0.03(1)
		3/3 x 3/4	3	26			15(13.0)				11(13.0)	0.62(1)
PGM(1)	ES	1/1 x 1/2	15	149	81(74.5)			68(74.5)				1.14(1)
		1/2 x 1/2	11	72	14(18.0)	25(18.0)		33(36.0)				3.65(2)
		1/2 x 2/2	8	80		40(40.0)		40(40.0)				0.00(1)
MDH(3)	BS	1/1 x 1/2	12	101	43(50.5)			58(50.5)				2.24(1)
		1/2 x 1/2	5	24	4(6.0)	7(6.0)		13(12.0)				0.01(1)
		1/2 x 2/2	8	73		36(36.5)		37(36.5)				0.01(1)
	ES	1/1 x 1/2	25	244	108(122.0)			136(122.0)				3.22(1)
		1/2 x 1/2	11	68	18(17.0)	20(17.0)		30(34.0)				1.05(2)
		1/2 x 2/2	6	59		33(29.5)		26(29.5)				0.83(1)
MDH(4)	BS	2/2 x 2/3	18	59		79(79.5)				80(79.5)		0.01(1)
		2/3 x 2/3	9	39		11(9.75)	6(9.75)			22(19.5)		2.14(2)
	ES	1/2 x 1/2	4	32	11(8.25)	12(8.25)		9(16.5)				4.41(2)
		1/2 x 2/2	12	114		55(57.0)		59(57.0)				0.14(1)

[a] BS = blue spruce; ES = Engelmann spruce.
[b] Progeny resulting from crosses between homozygous parents always bred true and therefore are not shown.
[c] G values corresponding to two significance levels and degrees of freedom are:
0.05 significance level: 3.84(1 df) and 5.99(2 df)
0.01 significance level: 6.64(1 df) and 9.21(2 df).

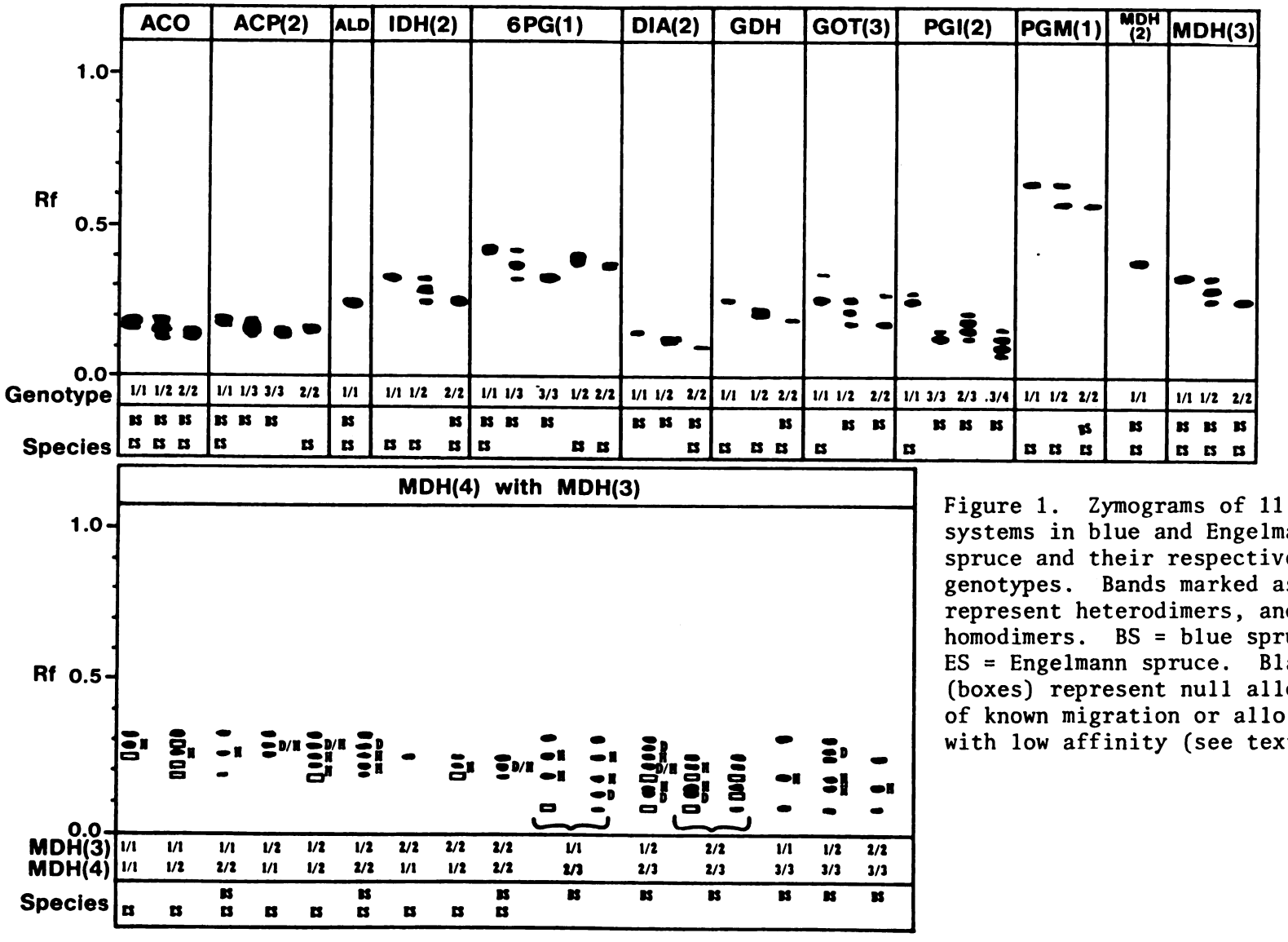


Figure 1. Zymograms of 11 enzyme systems in blue and Engelmann spruce and their respective genotypes. Bands marked as 'H' represent heterodimers, and as 'D' homodimers. BS = blue spruce; ES = Engelmann spruce. Blank bands (boxes) represent null allozymes of known migration or allozymes with low affinity (see text).

produced resolvable bands, and then only in megagametophyte and embryo tissue, but not in bud tissue. Therefore the inheritance of this enzyme system was inferred from segregation ratios of half-sib open-pollinated families from heterozygous mother trees instead of bud tissue from the seedling progeny.

In blue spruce, two allozymes were observed among the twenty parents in the mating study, and an additional null allele was found among the open-pollinated collections. The banding patterns observed in embryo tissue for both homozygotes and heterozygotes, except for individuals with null alleles, are shown in Figure 1. A single large, diffuse band of intermediate migration was observed in heterozygous embryo tissue for ACP(2) rather than the typical three-banded dimer pattern, possibly because there was so little migration distance between the two allozymes and staining for this enzyme system was relatively diffuse. The intermediate band of the heterozygous phenotype concurs with the dimeric structure of this enzyme as proposed for Norway spruce (P. abies (L.) Karst.) by Lundkvist (1975). The pooled segregation ratio of open-pollinated half-sib progeny from heterozygous females infers that ACP(2) is inherited as a one locus system (Table 4). Only two individuals (mother trees) from the open-pollinated collection possessed the null allele, and both of these parents were heterozygotes as determined by segregation of the haploid megagametophytes.

Table 4. Pooled segregation ratios observed in megagametophyte tissue of seed collected from heterozygous mother trees in the Dolores River drainage for two enzyme systems, and G-statistic from goodness-of-fit test.

Locus	Species[a]	Genotype	No. of Families	Observed Segregation	G(1 df)
ACP(2)	BS	1/3	15	64:67	0.07
		1/4	2	8:8	0.00
	ES	1/2	3	12:12	0.00
		2/4	4	18:14	0.50
DIA(2)	BS	1/2	21	88:92	0.09

[a] BS = blue spruce; ES = Engelmann spruce.

Among the 20 Engelmann spruce parents in the mating study, only one allozyme was observed, and it was intermediate in mobility to the two non-null allozymes observed in blue spruce (Figure 1). Among the open-pollinated collections, parents were found which possessed both the faster allozyme observed in blue spruce and a null allozyme. The pooled segregation ratio for these heterozygotes also infers ACP(2) is inherited as a one locus system (Table 4). It must be noted that mobility differences among the different ACP(2) allozymes in blue and Engelmann spruce are relatively small, and further study under a broader range of electrophoretic conditions may disclose even more alleles than observed in this study.

Aldolase

There were four zones of activity for all tissue types on gels stained for ALD, but only one zone produced clear bands. This zone was monomorphic among all parents of both species in the mating study (Figure 1; Table 3). Using the same buffer system among five white spruce (P. glauca (Moench) Voss) parents, Cheliak and Pitel (1984) observed a monomorphic zone at a similar migration distance. Also, Wendel and Parks (1982) observed three monomorphic zones for aldolase in Camelia japonica L.. The three non-resolvable zones in blue and Engelmann spruce all appear to be polymorphic, but unfortunately the staining in these zones was too diffuse to score reliably.

Isocitrate dehydrogenase

Three zones of activity were observed on gels stained for IDH. The fastest zone, IDH(1), did not produce clear bands in any tissue and therefore was not scored. The two slower zones, IDH(2) and IDH(3), were well resolved in megagametophyte, embryo and bud tissue. Two allozymes were observed at IDH(2) in Engelmann spruce (Figure 1), and they are inherited as a single locus (Table 3). The three-banded heterozygous phenotype suggests the functional form of IDH(2) is a dimer. These results are consistent with those presented for IDH in other conifer species (Cheliak and Pitel 1984; Neale et al. 1984; Neale and Adams 1981; O'Malley et al. 1979; Guries and Ledig 1978). Only one allozyme was observed among the 20 blue spruce parents, all being homozygous for the slower allozyme found in Engelmann spruce (Figure 1). Electromorphs in the slowest of the three zones, IDH(3), form heterodimers--as identified in haploid megagametophyte tissue--with electromorphs in the intermediate zone, IDH(2), but there were not enough individuals variable at IDH(3) to deduce a mode of inheritance.

6-phosphogluconate dehydrogenase

Two zones of activity were resolved for all tissue types on gels stained for 6PG. The slower zone appears to be controlled by two loci which form heterodimers much in the same manner as IDH(2) and IDH(3). However, there were

not enough individuals variable in this slower zone to determine the mode of inheritance.

Two bands were observed at the faster zone, 6PG(1), among the 20 blue spruce parents. The heterozygotes expressed a three-banded phenotype (Figure 1), indicating the functional form of 6PG(1) is a dimer. The distribution of progeny genotypes infers 6PG(1) is controlled as a single locus system (Table 3). These results are consistent with those presented for 6PG in other conifers (Cheliak and Pitel 1984; Neale et al. 1984).

Two bands were also observed at 6PG(1) among the 20 Engelmann spruce parents, but only the faster allozyme appears to be in common with that found in blue spruce. The slower variant in Engelmann spruce migrates somewhat faster than the slower allozyme in blue spruce (Figure 1). Based on heterozygote intermediacy, the functional 6PG(1) enzyme in Engelmann spruce is also a dimer, and the progeny distributions indicate a one locus system (Table 3). As observed for ACP(2), heterozygotes expressed a large, diffuse band rather than the typical three-banded dimer pattern, possibly because there was so little migration distance between the two allozymes and the relatively diffuse staining for this enzyme system.

Diaphorase

Several zones of activity were observed on gels stained for DIA, but only one zone, DIA(2), produced clear band patterns, and then only in megagametophyte and embryo

tissues. Therefore, the mode of inheritance of DIA(2) was determined from pooled segregation ratios of open-pollinated half-sib families from heterozygous mother trees.

Two variants were observed at DIA(2) among the 20 blue spruce parents, while Engelmann spruce was monomorphic for the slower allozyme (Figure 1). The pooled segregation ratio of half-sib families from heterozygous mother trees (Table 4) infers a single locus mode of inheritance for DIA(2). Heterozygotes were identified based on this segregation of the allozymes in haploid megagametophyte tissue. The heterozygote, as observed in embryo tissue, produces an intermediate phenotype relative to the two homozygotes, suggesting a multimeric structure for the functional DIA(2) enzyme. Reports of DIA in Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) (Neale et al. 1984; El-Kassaby et al. 1982) and Camelia japonica L. (Wendel and Parks 1982) have shown DIA to be a monomeric enzyme. Therefore the multimeric structure suggested by the band patterns observed in this study for DIA(2) may be incorrect. However, the intermediate band pattern is readily apparent, although diffuse, in diploid embryo tissue of blue and Engelmann spruce, so further study is warranted. Variability observed for DIA among full-sib progeny of white spruce was not heritable (Cheliak and Pitel 1984).

Glutamate dehydrogenase

One zone of activity was observed on gels stained for GDH, with good resolution for megagametophyte, embryo and bud tissues. Two variants were observed among the 20 Engelmann spruce parents, while only one allozyme--corresponding to the slower variant in Engelmann spruce--was observed in blue spruce (Figure 1). Distributions of the Engelmann spruce progeny infer GDH is inherited as a one locus system (Table 3). The heterozygous phenotype is intermediate in mobility and somewhat more diffuse relative to the two homozygotes, indicating GDH is functionally multimeric. Heterozygotes were also identifiable based on segregation of the allozymes in haploid megagametophyte tissue. Similar results have been reported for GDH in other conifers (Cheliak and Pitel 1984; Neale et al. 1984; Adams and Joly 1980; Mitton et al. 1979) and in maize (Pryor 1974).

Glutamate oxaloacetate transaminase

Three zones of activity were observed on gels stained for GOT. The two faster zones, GOT(1) and GOT(2), were observed across all tissue types, while the slowest zone, GOT(3), was best resolved in megagametophyte tissue, but also with good definition in embryo and bud tissue. There was insufficient variability in GOT(1) and GOT(2) among the 20 parental trees of either species to determine the mode of inheritance of these putative loci.

Triple-banded allozymes were observed at GOT(3) in blue and Engelmann spruce haploid megagametophyte tissue, the blue spruce phenotype migrating slower--approximately 0.1 R_f unit--relative to that of Engelmann spruce. Double-banded allozymes were observed at GOT(3) in embryo and bud tissue for both species (Figure 1). The double and triple-banded allozymes are apparently the product of a single allele, as they are inherited as a single unit. Possibly the multiple bands represent post-translational modification products of a single allozyme (Finnerty and Johnson 1979; Newton 1979). Double and triple-banded allozymes have been observed for GOT in eastern white pine (Pinus strobus L.) (Eckert et al. 1981), balsam fir (Abies balsamea (Linn.) Mill.) (Neale and Adams 1981), loblolly pine (P. taeda L.) (Adams and Joly 1980), pitch pine (P. rigida Mill.) (Guries and Ledig 1978), and Scotch pine (P. sylvestris L.) (Rudin and Ekberg 1978).

A single open-pollinated half-sib blue spruce family was heterozygous (segregating at a 5:3 ratio). Heterozygous embryos from this female produced a phenotype clearly indicating that GOT(3) is functionally dimeric, consistent with results reported for GOT in other species (Neale et al. 1984; El-Kassaby et al. 1982; Wendel and Parks 1982; O'Malley et al. 1979; Guries and Ledig 1978). Only the slower migrating bands of the double-banded allozymes stained well enough in the heterozygote to produce a clear dimer pattern (Figure 1). The staining of

the faster bands in the heterozygote was too diffuse to discern any band patterns.

Phosphoglucose isomerase

Gels stained for PGI exhibited two zones of activity, but the more anodal zone, PGI(1), did not produce sufficiently clear bands to score. The more cathodal zone, PGI(2), resolved well in all tissues, and exhibited three phenotypes among the 20 blue spruce parents but only one phenotype among the 20 Engelmann spruce parents. Three and four-banded allozymes were observed in megagametophyte tissue, while one, two and three-banded phenotypes were observed in embryo and bud tissue from homozygous individuals (Figure 1). As suggested for GOT(3), the multiple-banded allozymes for PGI(2) may be the result of post-translational modifications of these allozymes (Finnerty and Johnson 1979; Newton 1979). The multiple-banded nature of these allozymes were manifest in the dimers as well (Figure 1). The multibanded allozymes observed in this study for PGI(2) are consistent with those observed in Douoglas-fir (Neale et al. 1984).

The progeny distributions for PGI(2) in blue spruce infer a single locus mode of inheritance (Table 3). The heterozygous phenotypes (Figure 1) are consistent with reports for other species that PGI(2) is functionally dimeric (Cheliak and Pitel 1984; Neale et al. 1984; Adams and Joly 1980; Mitton et al. 1979; Guries and Ledig 1978).

Phosphoglucomutase

Two zones of activity were observed on gels stained for PGM, but only the fastest zone, PGM(1), was consistently resolvable among all three tissue types. The 20 Engelmann spruce parents expressed two alleles, and progeny distributions infer a one locus mode of inheritance for PGM(1) (Table 3). The heterozygous phenotype exhibits both bands found in the respective homozygous phenotypes (Figure 1), indicating that PGM(1) is functionally monomeric. These results are consistent with those reported for other conifers (Cheliak and Pitel 1984; Neale et al. 1984; Mitton et al. 1979).

Only one allozyme, the slower variant observed in Engelmann spruce, was observed among the 20 blue spruce parents. One blue spruce parent exhibited segregation of an electromorph somewhat intermediate to the two allozymes observed in Engelmann spruce, but was not expressed in embryo or bud tissue; the diploid phenotype resembled that of the other 19 blue spruce parents. Therefore this individual was scored as a homozygote corresponding to the slower allozyme.

Malate dehydrogenase

Four zones of activity were observed on gels stained for MDH, all zones equally resolvable in megagametophyte, embryo and bud tissues. The most anodally migrating zone, MDH(1), is double-banded, and only one of the Engelmann spruce parents segregated at this putative locus. It was

not possible to accurately determine the mode of inheritance of MDH(1) based on the progeny of only one individual and therefore will require further study. The second most anodal zone, MDH(2), was monomorphic among both sets of parents, and is represented by a single-banded phenotype (Figure 1). MDH(2) in blue and Engelmann spruce is similar to the MDH(1) locus as described for several other conifer speices by El-Kassaby (1981).

The third most anodal zone, MDH(3), was variable among both sets of parents. Progeny distributions of intraspecific crosses for both species show MDH(3) to be inherited as a single locus system (Table 3). The heterozygous phenotype exhibits three bands, including a band intermediate in migration to the two homozygotes (Figure 1), indicating MDH(3) is functionally dimeric in blue and Engelmann spruce. The banding patterns of MDH are relatively complicated because MDH(3) and MDH(4) form heterodimers (Figure 1), which is consistent with observations in other conifer species (El-Kassaby et al. 1982; El-Kassaby 1981; O'Malley et al. 1979; Guries and Ledig 1978).

The most cathodal zone, MDH(4), is the most variable and complicated of the four. Two allozymes were observed among the 20 Engelmann spruce parents, the most anodal of the two, MDH(4)-1, being null (Figure 1). The functional MDH(4) enzyme is also a dimer and forms heterodimers with the allozymes of the MDH(3) locus. However, in the

the allozymes of the MDH(3) locus. However, in the heterozygote MDH(4)-1/2, MDH(4)-2 appears to have a higher affinity for allozymes of the MDH(3) locus than for allozymes of the MDH(4) locus (Figure 1). Also, the higher affinity of MDH(4)-2 precludes MDH(4)-1 from forming a heterodimer with MDH(3) allozymes. Therefore, in MDH(4)-1/2 heterozygotes, MDH(4)-2 heterodimers apparently form at the expense of homodimers at this locus and MDH(4)-1 heterodimers.

The 20 blue spruce parents also expressed two alleles for MDH(4) (Figure 1), including the more cathodal allozyme found in Engelmann spruce, MDH(4)-2, and an even slower migrating allozyme, MDH(4)-3. Intralocus and interlocus interaction of MDH(4) heterozygotes results in a wide array of band patterns (Figure 1). Heterozygotes at this locus may express (i) only the heterodimer(s), (ii) the heterodimer(s) and intralocus dimer, or (iii) heterodimer(s), dimer and homozygous bands. As yet we have no explanation for the inconsistency in expression of the allozymes at this locus in the heterozygous condition for both blue and Engelmann spruce, but progeny distributions of both species for MDH(4) show it to be inherited as a single locus system (Table 3), indicating our interpretation of the phenotypes is probably correct.

There were no mobility differences observed among all tissue types for loci expressed in megagametophyte, embryo and bud tissues. The same observation was made in white

spruce (Cheliak and Pitel 1984), but mobility differences were observed for loci when expressed in embryo and needle tissue of Douglas-fir (Neale et al. 1984). The same extraction buffer was used for all tissues in the present study to minimize mobility differences due to preparative procedures.

A total of five electrophoresis buffers were screened before selecting the final buffers to be used in this study. The two selected buffers resulted in far superior resolution for a maximum number of enzyme systems. The remaining three buffers which were not reported in this study include a morpholine citrate buffer (Clayton and Tretiak 1972), another variation of the tris citrate/lithium borate buffer (Ridgway et al. 1970), and a tris citrate buffer (Nichols and Ruddle 1973).

A total of 26 different enzyme systems were screened initially in blue and Engelmann spruce, of which only the 13 listed produced consistent resolution. The remaining 13 enzymes which did not produce consistent resolution are alcohol dehydrogenase (ADH), fluorescent esterase (FLE), fructose-1,6-diphosphatase (FDP), fumarase (FUM), superoxide dismutase (SOD), glycerate-2-dehydrogenase (G2D), malic enzyme (ME), menadione reductase (MNR--the same as diaphorase, DIA), glutathione reductase (GLR--equivalent to the faster electromorphs of diaphorase), mannose-6-phosphate isomerase (MPI), sorbitol dehydrogenase

(SDH), uridine diphosphoglucose pyrophosphorylase (UDP), and glyceraldehyde-phosphate dehydrogenase (GPD). These are listed merely as background information for others interested in assaying these enzymes in blue and Engelmann spruce in hopes that resolution can be improved.

CHAPTER IV

ALLOZYME VARIATION OF BLUE AND ENGELMANN SPRUCE IN SOUTHWESTERN COLORADO

ABSTRACT

Open-pollinated single-tree cone collections of blue and Engelmann spruce were made in the Dolores River drainage in southwestern Colorado during the fall of 1983 from three elevational subpopulations of each species, including a zone where both species were present. Thirteen isozyme loci were assayed using megagametophytes from the half-sib seed collections. Fifty-four percent and 62 percent polymorphic loci (0.99 criterion) were observed for blue and Engelmann spruce, respectively. An average of 1.6 alleles per locus was observed in both species. Based on observed allele frequencies, average expected heterozygosities were 0.193 and 0.203 for blue and Engelmann spruce, respectively. Observed genotypic distributions at all loci conformed to Hardy-Weinberg expectations, indicating both populations are panmictic. Seventeen species-specific alleles were observed between blue and Engelmann spruce, and strong frequency differences were also observed between the two species at seven of the 13 loci.

Average genetic distance estimates indicated very little intraspecific genetic differentiation in the Dolores River drainage, while high degrees of divergence were observed between blue and Engelmann spruce. The greatest degree of genetic divergence between blue and Engelmann spruce was observed among the respective species subpopulations in the zone of overlap where both species were present. This suggests the two species do not hybridize naturally, or at least only very rarely (beyond the limits of the sample sizes in this study), because if they did this intermediate zone of overlap should indicate a convergence in their respective genetic compositions. The average genetic distance estimate between blue and Engelmann spruce (0.46) is comparable to sibling species or morphologically distinct species, indicating both prezygotic and postzygotic reproductive isolating mechanisms may be functioning to maintain the observed species integrity.

INTRODUCTION

Allozyme variation is readily observable and quantifiable at a large number of loci thanks to the rapid development of electrophoretic and associated biometrical techniques. Measures of isozyme variability and its partitioning--e.g., F-statistics (Wright 1951, 1965; Nei

1977) and genetic differentiation (Nei 1972, 1978; Rogers 1972)--are readily applicable to isozyme data and allow the observed variability within a species or between species to be partitioned according to various levels of population structure. These measures may also indicate how long two or more species have been separated phylogenetically (Sarich 1977) and the stage of speciation they exhibit (Ayala 1975, 1982).

The possibility of natural hybridization between blue and Engelmann spruce (Picea pungens Engelm. and P. engelmannii Parry ex Engelm., respectively) is interesting in regards to speciation and reproductive isolation in conifers. The two species are thought to be closely related phylogenetically (Daubenmire 1972; Nienstaedt and Teich 1972). While they are somewhat similar morphologically, a combination of traits will usually distinguish the two species (Schaefer and Hanover 1985a; Jones and Bernard 1977). There is some degree of elevational overlap in their respective habitats with ample opportunity for cross-pollination, and the results of many studies have suggested natural hybrids between blue and Engelmann spruce may exist, although no natural hybrids have been identified based on morphological and biochemical traits (e.g.: Habeck and Weaver 1969; Daubenmire 1972; Taylor et al. 1975; Mitton and Andalora 1981; Schaefer and Hanover 1985a and b). A few artificial hybrids have been

produced and indicated the crossability between the two species is very low (Fechner and Clark 1969; Kossuth and Fechner 1973). Isozymes represent more closely the variability at the DNA level relative to morphological and biochemical traits and therefore may be more useful in distinguishing the two species and their hybrids. The objective of this study was to describe and quantify the observed variability among 13 loci both within and between blue and Engelmann spruce in a drainage in southwestern Colorado as part of a broader study to determine if natural interspecific hybrids do exist.

MATERIALS AND METHODS

During the fall of 1983, open-pollinated cones were collected from individual blue and Engelmann spruce trees in the Dolores River drainage in southwestern Colorado. The Dolores River drainage encompasses elevationally the habitats of both blue and Engelmann spruce and there exist many sites in the drainage where both species occur in close proximity. The drainage is easily accessible, and genetic studies investigating morphological and terpenoid variability of the two species have been conducted previously in the study area (Hanover 1975; Reed and Hanover 1983; Schaefer and Hanover 1985a and b).

Before collections were made in 1983, the Dolores River and some of its tributaries were subjectively divided into five elevational species-occupation zones: Zone 1 - the zone of lowest elevation, extending from 2225 to 2400 meters (m), and in which blue spruce occurs but Engelmann spruce does not; Zone 2 - extending from 2400 to 2590 m in elevation, and where blue spruce is almost exclusively predominant relative to the occurrence of Engelmann spruce, but scattered individuals of Engelmann spruce are present on the adjacent hillsides of north aspect; Zone 3 - an elevationally intermediate zone relative to the habitats of blue and Engelmann spruce, extending from 2590 to 2770 m, and where both species are present and often in close proximity; Zone 4 - extending from 2770 to 2960 m in elevation, and where Engelmann spruce is almost exclusively predominant but with a few blue spruce individuals present, primarily on hillsides of south aspect; Zone 5 - the zone of highest elevation, extending from 2960 to 3140 m and in which Engelmann spruce is present but blue spruce is not. The breakdown of single-tree collections made in each zone during the fall of 1983 is as follows:

Species	Zone 1	Zone 2	Zone 3	Zone 4	Zone 5	Total
Blue spruce	16	22	18			56
Engelmann spruce			23	31	22	76

The cones were kept separate by mother tree and taken back to the nursery to be dried and the seed extracted and

blown. Collections were made in all five zones along the Dolores River and at a site in zone 3 of Scotch Creek, a tributary of the Dolores River. Both blue and Engelmann spruce occur at the Scotch Creek site, often side-by-side, and pollen shed and female strobilus receptivity are coincident among both species in the spring.

Isozymic genotypes of the mother trees were determined using haploid megagametophyte tissue from germinated seed, eight megagametophytes per parent. This sample size gives a probability of $(1/2)^7 = 0.0078$ of misclassifying a heterozygote at a given locus. The inheritance of the respective loci are described elsewhere (Ernst et al. 1985a). The individual megagametophytes were placed in 0.5ml sample vials, and these were put in foam storage blocks, wrapped in plastic wrap, and frozen at -20°C until used.

Electrophoretic conditions were as described elsewhere (Ernst et al. 1985a). A total of 13 loci from 11 enzyme systems were analyzed: Aconitase (ACO), acid phosphatase (ACP(2)), isocitrate dehydrogenase (IDH(2)), 6-phosphogluconate dehydrogenase (6PG(1)), diaphorase (DIA(2)), glutamate dehydrogenase (GDH), glutamate oxaloacetate transaminase (GOT(3)), phosphoglucose isomerase (PGI(2)), phophoglucomutase (PGM(1)), and malate dehydrogenase (MDH(2), MDH(3), and MDH(4)).

Prior to electrophoresis, the sample vials were removed from the freezer and placed in a plexiglass block

embedded in ice. Two drops of extraction buffer (Wendel and Parks 1982) were placed in each vial and the megagametophyte homogenized using a motor-driven teflon grinding tip. Filter paper wicks (Whatman, No. 3) were used to absorb the homogenate and these were inserted at the vertically sliced gel origin.

Multiple locus enzyme systems were scored with the fastest--most anodally--migrating zone labeled as locus 1, next fastest as locus 2, etc. Multiple allozymes at a locus were numbered in the same manner, with the fastest allozyme labeled as allele 1, etc.. Mobilities of the different allozymes presented here have been quantified--R_f-values--and are presented elsewhere (Ernst et al. 1985a) as diagramatic zymograms of the loci as expressed in diploid tissue.

Allele frequencies were determined for each of the six subpopulations, three for each species, and for the species groups in total. Observed genotypic ratios were compared to those expected under Hardy-Weinberg equilibrium conditions using the log-linear G statistic (Sokal and Rohlf 1969). Expected heterozygosities for each locus corrected for small sample size, average expected heterozygosities and their standard errors, genetic distance estimates for small sample sizes and their standard errors (Nei 1972, 1978; Nei and Roychoudhury 1974), and Rogers' coefficient of distance (Rogers 1972)

were computed using the program written by Dowling and Moore (1984).

RESULTS

Of the 13 loci surveyed among the two species in the Dolores River drainage, seven were found to be polymorphic in blue spruce and eight in Engelmann spruce (Table 1). Only two loci, ALD and MDH(2), were monomorphic across both species. The remainder of the monomorphic loci within each species were either fixed for opposing alleles--GOT(3)--or fixed in one species and polymorphic in the other--IDH(2), DIA(2), GDH, PGI(2), PGM(1)--based on a presence frequency of 0.01 or greater (0.99 criterion). Rare alleles were observed which would render some of these loci polymorphic at less restrictive criteria (Table 1), but the two species are still quite different in allelic composition at these and other loci. For example, strong frequency differences occur between the two species at ACP(2), where allele 1 predominates in blue spruce but is somewhat rare in Engelmann spruce, while allele 2 predominates in Engelmann spruce but was not observed in blue spruce (Table 1). The average number of alleles per locus and percent polymorphic loci at 0.99 and absolute criterion are listed in Table 1.

Blue spruce possessed a greater number of rare alleles--frequency less than 0.01--than Engelmann spruce

Table 1. Allelic frequencies and expected heterozygosities (corrected for small sample size) among the respective loci and overall for blue and Engelmann spruce open-pollinated collections in the Dolores River drainage in southwestern Colorado (0.99 criterion).

Locus	Species[a]	No. Families	Allele 1	2	3	4	H	Std. error
ACO	BS	56	0.652	0.348			0.458	
	ES	76	0.461	0.539			0.499	
ACP(2)	BS	56	0.77	-----	0.205	0.018[b]	0.357	
	ES	76	0.033	0.941	-----	0.026	0.114	
ALD	BS	56	1.000				0.000	
	ES	76	1.000				0.000	
IDH(2)	BS	54	-----	1.000	*[c]---		0.000	
	ES	76	0.165	0.835	-----		0.277	
6PG(1)	BS	55	0.786	-----	0.214		0.340	
	ES	75	0.900	0.100	-----		0.181	
DIA(2)	BS	55	0.282	0.718			0.408	
	ES	76	-----	1.000			0.000	
GDH	BS	55	-----	1.000	*----		0.000	
	ES	76	0.730	0.270	-----		0.397	
GOT(3)	BS	53	*----	1.000	*----		0.000	
	ES	76	1.000	-----	-----		0.000	
PGI(2)	BS	55	-----	0.036	0.964	*----	0.071	
	ES	74	1.000	*----	-----	-----	0.000	
PGM(1)	BS	56	-----	1.000			0.000	
	ES	76	0.592	0.408			0.486	
MDH(2)	BS	56	1.000				0.000	
	ES	76	1.000				0.000	
MDH(3)	BS	56	0.446	0.554	-----		0.499	
	ES	74	0.689	0.311	*----		0.431	
MDH(4)	BS	54	*----	0.750	0.250		0.378	
	ES	73	0.144	0.856	-----		0.248	
Average	BS	55.2					0.193	0.058
	ES	75.4					0.203	0.055

Average no. alleles per locus			Percent polymorphic loci		
	Criterion			Criterion	
	0.99	Absolute		0.99	Absolute
BS	1.62	2.08	BS	54%	77%
ES	1.69	1.85	ES	62%	69%

[a] BS = blue spruce; ES = Engelmann spruce.
[b] Null allele.
[c] * designates an allele present at a frequency less than 0.01 (0.99 criterion).

among the individuals surveyed in the Dolores River drainage (Table 1). The slight disparity in sample sizes between the two species groups may account for some of this difference, although the larger sample size for Engelmann spruce should result in a greater number of rare alleles sampled relative to blue spruce.

Expected heterozygosities ranged from 0.000 to 0.499 among the loci of the two species groups, with Engelmann spruce only slightly more variable than blue spruce at the 0.99 criterion (Table 1). Observed genotypic frequencies at the polymorphic loci of the two species did not deviate significantly from those expected, indicating the two populations conform to Hardy-Weinberg equilibrium. When broken down into subpopulations (by zone, Tables 2 and 3), observed genotypic frequencies did deviate significantly from those expected for the ACO locus in the zone 2 subpopulation of blue spruce and the MDH(3) locus in the zone 5 subpopulation of Engelmann spruce. However, these were the only significant departures from Hardy-Weinberg equilibrium among the loci of the respective subpopulations.

Some elevational trends were evident in allelic frequencies among the subpopulations (Tables 2 and 3). In blue spruce, a fairly marked elevational change occurs for DIA(2) (Table 2). More subtle elevational changes occur for ACO, 6PG(1) and MDH(3). Expected heterozygosities of

Table 2. Allelic frequencies and expected heterozygosities (corrected for small sample size) for the seven polymorphic loci observed among three blue spruce subpopulations from the Dolores River drainage in southwestern Colorado.

Locus	Zone[a]	No. Families	Allele[b] 1	2	3	4	H	error
ACO	1	16	0.750	0.250			0.387	
	2	22	0.591	0.409			0.495[d]	
	3	18	0.639	0.361			0.475	
ACP(2)	1	16	0.781	-----	0.188	0.031	0.365	
	2	22	0.682	-----	0.273	0.023	0.471	
	3	18	0.861	-----	0.139	-----	0.246	
6PG(1)	1	16	0.750	-----	0.250		0.387	
	2	22	0.795	-----	0.205		0.334	
	3	18	0.833	-----	0.167		0.286	
DIA(2)	1	16	0.313	0.687			0.444	
	2	22	0.364	0.636			0.474	
	3	18	0.750	0.250			0.386	
PGI(2)	1	16	-----	0.031	0.969		0.062	
	2	21	-----	0.048	0.952	*[c]---	0.094	
	3	18	-----	0.028	0.972		0.056	
MDH(3)	1	16	0.375	0.625			0.484	
	2	22	0.455	0.545			0.499	
	3	18	0.500	0.500			0.500	
MDH(4)	1	16	*----	0.800	0.200		0.330	
	2	22	-----	0.727	0.273		0.406	
	3	17	-----	0.735	0.265		0.401	
Average[e]	1	15.9					0.189	0.057
	2	21.9					0.214	0.064
	3	17.9					0.182	0.057

[a] See text.
[b] Alleles shown based on 0.99 criterion for total species sample.
[c] * designates an allele present at a frequency less than 0.01 in the total species sample.
[d] Only for ACO (zone 2) did genotypic ratios deviate significantly (0.05) from those expected.
[e] Includes monomorphic loci.

Table 3. Allelic frequencies and expected heterozygosities (corrected for small sample size) for the eight polymorphic loci observed among three Engelmann spruce subpopulations from the Dolores River drainage in southwestern Colorado.

Locus	Zone[a]	No. Families	Allele[b] 1	2	3	4	H	error
ACO	3	23	0.543	0.457			0.499	
	4	31	0.468	0.532			0.499	
	5	22	0.364	0.636			0.474	
ACP(2)	3	23	-----	1.000	-----	-----	0.000	
	4	31	-----	0.968	-----	0.032	0.063	
	5	22	0.114	0.814	-----	0.045	0.284	
IDH(2)	3	23	0.109	0.891			0.199	
	4	31	0.210	0.790			0.337	
	5	22	0.159	0.841			0.274	
6PG(1)	3	22	0.932	0.068			0.130	
	4	31	0.871	0.129			0.228	
	5	22	0.909	0.091			0.169	
GDH	3	23	0.696	0.304			0.433	
	4	31	0.710	0.290			0.419	
	5	22	0.795	0.205			0.337	
PGM(1)	3	23	0.630	0.370			0.477	
	4	31	0.661	0.339			0.456	
	5	22	0.455	0.545			0.499	
MDH(3)	3	22	0.682	0.318			0.444	
	4	30	0.800	0.200	*[c]---		0.325	
	5	22	0.545	0.455			0.499[d]	
MDH(4)	3	21	0.143	0.857			0.251	
	4	31	0.129	0.871			0.228	
	5	21	0.167	0.833			0.285	
Average[e]	3	22.5					0.188	0.058
	4	30.9					0.197	0.054
	5	21.9					0.218	0.057

[a] See text.
[b] Alleles shown based on 0.99 criterion for total species sample.
[c] * designates an allele present at a frequency less than 0.01 in the total species sample.
[d] Only for MDH(3) (zone 5) did genotypic ratios deviate significantly (0.05) from those expected.
[e] Includes monomorphic loci.

the Engelmann spruce subpopulations tended to be more variable with increasing elevation (Table 3). For Engelmann spruce, consistent elevational shifts in allelic frequency were observed at ACO and GDH (Table 3).

Nei's (1978) and Rogers' (1972) measures of genetic distance are presented in Table 4. While the relative values within each are essentially equivalent between the two techniques, the Nei estimates are consistently greater than the Rogers estimates. This was expected based on the results of other empirical studies (Futuyma 1979). The large values obtained for comparisons among blue and Engelmann spruce subpopulations and between the two species groups (Table 4) indicate the strong genetic divergence of these two species which are thought to be closely related and possibly hybridize naturally. Distances between subpopulations within each species are very small, with the estimates exceeded by their respective standard errors (Table 4).

DISCUSSION

The observed percentages of polymorphic loci (0.99 criterion) of 54 percent and 62 percent, respectively, for blue and Engelmann spruce correspond well to a mean value of 67 percent reported in a survey of 20 conifer species (Hamrick et al. 1981). In the present study, 13 loci were

Table 4. Nei's (1978) corrected genetic distance estimates (below diagonal) and their standard errors (in parentheses), and Rogers' (1972) distance coefficients (above diagonal) among the six blue and Engelmann spruce subpopulations.

	BS 1	BS 2	BS 3	ES 3	ES 4	ES 5
BS 1[a]		0.0039	0.0687	0.4274	0.4513	0.4183
BS 2	0.0000 (0.0216)		0.0541	0.4112	0.4358	0.4020
BS 3	0.0155 (0.0221)	0.0109 (0.0189)		0.4432	0.4685	0.4342
ES 3	0.4671 (0.2024)	0.4603 (0.2013)	0.5308 (0.2191)		0.0343	0.0638
ES 4	0.4993 (0.2054)	0.4866 (0.2034)	0.5587 (0.2210)	0.0000 (0.0098)		0.0691
ES 5	0.4444 (0.1941)	0.4338 (0.1937)	0.5057 (0.2095)	0.0052 (0.0117)	0.0089 (0.0122)	

[a] BS = blue spruce; ES = Engelmann spruce; numerals are zone designations.

assayed among blue and Engelmann spruce individuals comprising essentially single populations of each species. The individual studies which made up the survey (Hamrick et al. 1981) assayed an average of 20 loci per species and sampled anywhere from one to 34 populations. In general, higher amounts of isozyme variability have been observed in conifers than in dicots or monocots, possibly because of the life history characteristics of conifers (Hamrick et al. 1981; Shaw and Allard 1981). Conifers rely primarily on outcrossing and are relatively intolerant of selfing, rely upon wind for pollination and seed dispersal, exhibit high fecundity, are generally widespread in distribution and have long generation intervals. The same survey (Hamrick et al. 1981) reported an average of 2.2 alleles per locus among the 20 conifer species, slightly greater than the 1.6 alleles per locus observed in this study for both blue and Engelmann spruce.

The average expected heterozygosities--the average number of heterozygous loci per individual--of 0.193 for blue spruce and 0.203 for Engelmann spruce were higher than some estimates reported for other conifers; 0.116 for lodgepole pine (Pinus contorta Dougl.) (Wheeler and Guries 1982), 0.157 for Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) (Yeh and O'Malley 1980), 0.146 for pitch pine (P. rigida Mill.) (Guries and Ledig 1981), 0.123 for ponderosa pine (P. ponderosa Dougl. ex Laws.) (O'Malley et al. 1979)

and 0.147 for Sitka spruce (Picea sitchensis (Bong.) Carr.) (Yeh and El-Kassaby 1979). The slightly higher estimates for blue and Engelmann spruce may be due to the fewer number of loci sampled in this study (13) relative to those cited above (20 to 42 loci) (Leigh Brown and Langley 1979), the number of progeny analyzed per tree and the number of trees sampled per population (Morris and Spieth 1978), or simply because blue and Engelmann spruce are more variable. As mentioned previously, the percentage of polymorphic loci observed in this study was consistent with other conifer species, and the number of alleles per locus was less than generally reported elsewhere. Therefore, the loci sampled in this study must be nearer to allelic equilibrium--frequencies approaching 0.5 in a two allele system, with associated higher expected heterozygosity--than those assayed in other species; i.e., fewer "common" alleles. Blue spruce may exhibit even more "latent" variability than Engelmann spruce based on the large number of rare alleles observed in this species.

The high degree of conformity to Hardy-Weinberg equilibrium expectations among the sampled loci suggests the two species and the sampled subpopulations are randomly mating with sufficient amounts of gene flow to minimize the effects of genetic drift or selection. This appears to be true in general for conifers (Wheeler and Guries 1982), possibly because of their life history characteristics (Hamrick et al. 1981), as mentioned previously. Only

slight trends were observed between subpopulations within each species at the individual loci (Tables 2 and 3), but Engelmann spruce subpopulations did exhibit greater heterozygosities with increasing elevation, indicating the higher elevation populations of this species may be more diverse.

Blue and Engelmann spruce in the Dolores River drainage possess some marked differences in allelic frequencies and even some species-specific alleles. GOT(3) was essentially fixed for opposing alleles between the two species, although one blue spruce individual was heterozygous for both alleles. The alleles coding for the slower allozymes at IDH(2), GDH, and PGM(1) were essentially fixed in blue spruce, while these loci were variable in Engelmann spruce, and at GDH and PGM(1) the allele observed in blue spruce was not the more common allele found in Engelmann spruce (Table 1). In Engelmann spruce, DIA(2) was fixed for the allele responsible for the slower allozyme observed in the variable blue spruce population. PGI(2) also presents an essentially oppositely-fixed situation, where blue spruce possess three alleles--PGI(2)-2,3,4--and Engelmann spruce is essentially fixed for PGI(2)-1, with one Engelmann spruce individual found to be heterozygous for alleles 1 an 2 (Table 1). In both instances where blue and Engelmann spruce possess essentially oppositely-fixed alleles, the two observed

heterozygous individuals --one individual heterozygous for GOT(3)-1/2 and the other heterozygous for PGI(2)-1/2--did not possess at any other loci alleles characteristic of the other species. Whether or not these two individuals were backcrossed hybrids or pure species could not be determined based on the number of loci and number of individuals sampled in this study.

Other loci contained species-specific alleles, with varying degrees of diagnostic value. For example, Engelmann spruce possesses a very common and unique allele at ACP(2) (Table 1). Also, other species-specific alleles with lower frequencies were observed at ACP(2), 6PG(1), GDH, GOT(3), MDH(3), and MDH(4) (Table 1). A small survey of 24 blue spruce individuals from outside the Dolores River drainage--six from South Park, Colorado, eight from the White River National Forest in Colorado, and ten individuals from the Lincoln National Forest in New Mexico--did not reveal any alleles not observed in the Dolores River collections (unpublished data).

The genetic distance statistic developed by Nei (1972, 1978) estimates the accumulated number of detectable gene substitutions per locus among the sampled populations. The intraspecific subpopulation comparisons indicated very little genetic differentiation within the two species in the Dolores River drainage. However, high degrees of divergence were observed for the interspecific subpopulation comparisons and between the two species as a

whole in the sample area. The average number of allelic substitutions per locus between the two species as measured in this study was 0.46, or 46 complete allelic substitutions for every 100 gene loci. The zone 3 subpopulation of blue spruce--the zone in which both blue and Engelmann spruce are common--exhibited the greatest degree of genetic divergence from Engelmann spruce subpopulations, while the zone 4 Engelmann spruce subpopulation exhibited a slightly greater degree of divergence from the blue spruce subpopulations than did the zone 3 subpopulation. These trends are of interest, because if blue and Engelmann spruce do hybridize naturally, their zone of overlap should show the least amount of divergence because of shared genes. These statistics indicate there may be selection for just the opposite, where blue and Engelmann spruce species identities are even stronger in the zone of overlap than in the peripheral zones.

The average genetic distance estimate of 0.46 between blue and Engelmann spruce, computed using allelic frequencies calculated at the 0.99 criterion, is comparable to average genetic distance estimates observed among sibling species--species which are morphologically similar but quite distinct genetically and are reproductively isolated--and morphologically distinct species across a wide variety of organisms (Ayala 1975, 1982). The results

of this study indicate that blue and Engelmann spruce should be strongly reproductively isolated, possessing both prezygotic and postzygotic reproductive isolating mechanisms (Ayala 1982), and will maintain their species identities even in the presence of the other species. This supports the poor crossability, prezygotic incompatibility and hybrid inviability reported earlier for interspecific crosses between blue and Engelmann spruce (Fechner and Clark 1969; Kossuth and Fechner 1973).

CHAPTER V

ASSESSMENT OF NATURAL HYBRIDIZATION AND INTROGRESSION BETWEEN BLUE AND ENGELMANN SPRUCE IN SOUTHWESTERN COLORADO

ABSTRACT

In a partial diallel mating design among 20 blue and 20 Engelmann spruce parents, the interspecific cross was successful only with Engelmann spruce as the female parent. No viable seed were obtained from the reciprocal cross among the 60 full-sib families attempted. Under the conditions of artificial pollination and a controlled germination environment, very low interspecific crossability was observed, with an average of 0.3 percent germinated seed on a total seed basis across all 20 Engelmann spruce females. Many abnormalities were observed among the hybrid germinants, suggesting hybrid inviability also contributes to the low crossability between these two species.

Isozyme analysis can be used as evidence for interspecific hybridization between blue and Engelmann spruce because of the unique genotypic compositions of the hybrids relative to the two species. No natural F_1 hybrids between blue and Engelmann spruce were observed in this study based on isozyme analysis of mature individuals or their seedling progeny. Backcrossed hybrids may exist, but determination of such was beyond the resolution of this

study based on the number of loci and number of individuals sampled. Analyses included samples of open-pollinated seed from blue and Engelmann spruce females located in an area where both species are present in close proximity--often side-by-side--and flowering phenology is coincident between the two species. The probability of finding a mature natural hybrid must be very small due to intraspecific pollen competition, incompatibility, hybrid inviability and the environmental conditions imposed upon the rare viable interspecific seed during germination, seedling establishment and growth in the field.

INTRODUCTION

Reproductive isolation is the primary criterion for the definition of biological species, each species representing an independent and discrete evolutionary entity (Ayala 1982). Reproductive isolation may develop as a by-product of evolutionary divergence when two incipient species are separated geographically, or possibly while they are in sympatry through mutations which prevent cross-compatibility but maintain self-compatibility. Intraspecific crossability differences also exist in many organisms due to a wide variety of causes.

Blue and Engelmann spruce (Picea pungens Engelm. and P. engelmannii Parry ex Engelm., respectively) represent an

interesting species combination in regards to reproductive isolation and speciation. Studies of morphological and chemical variability among blue and Engelmann spruce have suggested the two species are phylogenetically closely related, blue spruce possibly the result of a single speciation event from the older Engelmann spruce (Daubenmire 1972; Nienstaedt and Teich 1971; Taylor et al. 1975). The two species are morphologically quite similar, although a combination of traits will generally distinguish the two species (Schaefer and Hanover 1985a; Jones and Bernard 1977). They are both montane species and occupy overlapping habitats in western North America. Blue spruce is primarily a riparian species, found along streams and adjacent hillsides at elevations of 2000 to 3000 meters. Engelmann spruce is generally found above this elevation, occupying upper valleys, hillsides and plateaus in pure stands or mixed with subalpine fir (Abies lasiocarpa (Hook.) Nutt.). In elevationally intermediate zones, the two species can often be found in close proximity with ample opportunity for cross-pollination. In these intermediate zones, there is enough overlap between blue and Engelmann spruce in phenology of pollen shed and female strobilus receptivity for natural cross-pollination to occur (Fechner and Clark 1969; Ernst, unpublished data). Some individuals of intermediate phenotype between the two species have been identified based on morphological and

biochemical traits (Daubenmire 1972; Taylor et al. 1975; Schaefer and Hanover 1985a and b). A few artificial hybrids between blue and Engelmann spruce have been produced, and interspecific crossability was very low (Fechner and Clark 1969; Kossuth and Fechner 1973), but only one or two parents of each species were used in the hybridizations.

The objectives of this study were to (1) produce known hybrids between blue and Engelmann spruce, (2) quantify the crossability between the two species using a large number of parents, (3) determine if isozyme analysis can be used to identify blue-Engelmann hybrids, and (4) determine if hybrids between blue and Engelmann spruce exist in nature.

The Dolores River drainage in southwestern Colorado was chosen as the study site for two primary reasons. First, there are many sites within the drainage where both blue and Engelmann spruce are present, and at these sites pollen flow and female strobilus receptivity are coincident between the two species. Also, studies investigating the genetic variability in morphological and terpenoid characters of blue and Engelmann spruce have previously been conducted in this drainage (Hanover 1975; Reed and Hanover 1983; Schaefer and Hanover 1985a and b).

Blue and Engelmann spruce differ markedly in their isozymic compositions (Ernst et al. 1985b). Several species-specific alleles were observed, and strong frequency differences were found between the two species at

seven of the 13 loci analyzed. Therefore F_1 hybrids between blue and Engelmann spruce, if they can be found in nature or artificially produced, should exhibit unique isozyme genotypes relative to the two species. However, backcrossed hybrids may not be identifiable based on an analysis of 13 enzymatic loci unless very large sample sizes are obtained.

MATERIALS AND METHODS

The Dolores River and five of its tributaries were divided elevationally into five species-occupation zones. Zone 1, the zone of lowest elevation and extending from 2225 to 2400 meters (m), was a "pure" blue spruce zone relative to the occurrence of Engelmann spruce. Zone 2, extending form 2400 to 2590 m, was almost exclusively blue spruce in composition with a few scattered Engelmann spruce individuals. Zone 3, extending from 2590 to 2770 m, was an elevationally intermediate zone relative to the habitats of blue and Engelmann spruce, with both species present and often in close proximity. Zone 4, extending from 2770 to 2960 m in elevation, was occupied primarily by Engelmann spruce with a few scattered blue spruce individuals present. Zone 5, the zone of highest elevation and extending from 2960 to 3140 m, was a "pure" Engelmann

spruce zone. The parents used to make the interspecific matings were located in zones 2, 3 and 4--ten blue spruce individuals from each of zones 2 and 3, and ten Engelmann spruce individuals from each of zones 3 and 4, for a total of 40 parents, 20 of each species. The 40 parents were selected primarily on the basis of fecundity and climbability. All parents were readily identifiable as to species and no putative hybrids were found in any of the zones along the Dolores River.

The partial diallel mating design used in this study was comprised of three intraspecific matings--including selfs--and three interspecific matings per parent. The results of the intraspecific matings and details of the pollination and cone collection procedures are described elsewhere (Ernst et al. 1985). Each biparental interspecific cross was replicated three times on a female parent. The pollinations were carried out during the spring of 1983 using fresh pollen. In the fall of 1983, the control-pollinated seed was collected and kept separate by isolation bag. During this time single-tree open-pollinated cone collections were also made from each of the 40 parents in the mating design and also from nine blue spruce and 11 Engelmann spruce individuals in zone 3 of Scotch Creek, a tributary of the Dolores River. The Scotch Creek site serves as a putative hybrid swarm area, as both blue and Engelmann spruce are present, often side-by-side, and pollen shed and female strobilus receptivity occur

simultaneously among both species in the spring. Most individuals at the Scotch Creek site were readily identifiable as to species. In the fall of 1984, dormant vegetative buds were collected from each of the 40 parents used in the mating design and stored at -20°C until used in the electrophoretic analysis.

The open and control-pollinated cones were dried, the number of cones per accession recorded, and the seed extracted by hand and blown to separate empty and putatively full seed. For the control-pollinated accessions, the number of cones per bag damaged by insects was also recorded, and both empty and putatively full seed were counted separately. The seed was kept in cold storage (4°C) until used.

Germination tests were conducted during the summer of 1984 using a maximum of 30 seed per isolation bag, depending on availability of seed per bag. The number of newly germinated seed was recorded daily, and the germinants were then planted in individual plant bands in the greenhouse. Germination was considered complete after 30 days, and the number of ungerminated seed were recorded and then each was dissected to determine the number of full but ungerminated seed versus empty seed. The percent germinated and percent ungerminated-but-full seed were determined from the germination test and then extrapolated to a total seed basis--full and empty--to serve as the

dependent variables in the analysis. Details of the germination procedures and results of the intraspecific progeny are given elsewhere (Ernst et al. 1985). Percent germination was used as the measure of interspecific crossability because it estimates the number of viable seed or progeny produced for a given cross. Percent ungerminated-but-full seed was measured primarily to detect postzygotic abnormalities.

The model equation used to estimate the fixed and random effects for the germination data and its associated assumptions are given elsewhere (Ernst et al. 1985). Using best linear unbiased prediction (BLUP) techniques (Mao 1982), parental general combining ability (GCA) estimates and individual-cross specific combining ability (SCA) estimates were determined. From these, restricted maximum likelihood (REML) techniques (Schaeffer 1976) were used to estimate the GCA, SCA and error variances. Under the assumptions that the blue and Engelmann spruce populations were sampled at random, each is randomly mating, and there is no inbreeding, epistasis or linkage, the GCA variance (σ^2_G) corresponds to one-fourth the additive variance ($1/4\sigma^2_A$), and the SCA variance (σ^2_S) corresponds to one-fourth the nonadditive (dominance) variance ($1/4\sigma^2_D$) for the trait in the partial diallel mating design (Kempthorne and Curnow 1961).

The seedlings from the germination test were grown under accelerated-optimal-growth conditions (Hanover et al.

1976) in the greenhouse from August, 1984, until January, 1985, when the seedlings were allowed to go dormant. Dormant vegetative buds were collected from each of the seedling progeny in March, 1985, and stored at -20°C until used in electrophoresis.

Nine enzyme systems were assayed in the electrophoretic analysis (Table 1). Genotypes of the parents in the controlled pollinations were determined by simultaneous comparison of isozymes in bud, embryo and megagametophyte tissues. Progeny genotypes were characterized using dormant vegetative bud tissue. The preparatory techniques and electrophoretic conditions utilized in this study are reported elsewhere (Ernst et al. 1985a), including the inheritance of the 11 loci from the nine enzyme systems analyzed in this study. For multiple locus enzyme systems, the fastest migrating--most anodal--zone was designated as locus 1, the next fastest 2, etc. Multiple allozymes within each locus were numbered in the same manner, with the fastest allozyme labeled as allele 1, etc. Mobilities of the different allozymes were quantified relative to the buffer front (R_f). Where possible, segregation tests of observed progeny genotypes were made using the log-linear G-statistic (Sokal and Rohlf 1969).

The single-tree open-pollinated collections from zone 3 of Scotch Creek were analyzed to determine if any blue-Engelmann hybrid progeny could be identified

Table 1. The eleven loci from nine enzyme systems analyzed among the Engelmann x blue spruce hybrids. The numbers in parentheses represent locus designations.

Enzyme	Abbreviation	E.C. No.
Aconitase	ACO	4.2.1.3
Aldolase	ALD	4.1.2.13
Isocitrate dehydrogenase	IDH(2)	1.1.1.42
Malate dehydrogenase	MDH(2)	1.1.1.37
	MDH(3)	
	MDH(4)	
6-phosphogluconate dehydrogenase	6PG(1)	1.1.1.44
Glutamate dehydrogenase	GDH	1.4.1.3
Glutamate oxaloacetate transaminase	GOT(3)	2.6.1.1
Phosphoglucose isomerase	PGI(2)	5.3.1.9
Phosphoglucomutase	PGM(1)	2.7.5.1

electrophoretically. Up to 40 embryos from partially germinated seeds were analyzed from each female parent.

RESULTS

Full-sib interspecific hybrids were obtained from the controlled pollinations, but only with Engelmann spruce as the female parent. Much lower crossabilities--i.e., fewer germinated seed--were observed for the hybrid crosses than for the intraspecific crosses (Table 2). Means of interspecific full-sib family viable seed yields ranged from 0.00 to 1.74 percent, with an overall mean of 0.30 percent. Of a total 60 possible full-sib Engelmann x blue spruce--female x male--families, only 30 families produced hybrid progeny. Sixteen of the 20 Engelmann spruce females combined with 17 of the 20 blue spruce male parents to produce a total of 158 viable hybrid progeny among the 30 full-sib families. The interspecific crosses also resulted in a much higher proportion of abnormal--ungerminated-but-full--seed (Table 2). This is not surprising, as many abnormalities were observed among the viable hybrid seedlots. These included a very high frequency of multiple embryony, fused multiple embryos, and a very high incidence of reverse germination. Up to six embryos germinated from a single seed, with two or three embryos per seed very common, while no multiple embryos were observed among the

intraspecific controlled crosses. Abnormalities and electrophoretic analysis were used to verify the hybridity of the interspecific crosses. Of a total 360 interspecific full-sib replicates attempted in the study, nine replicates were observed to be contaminated with very small amounts of intraspecific pollen based on isozyme genotypes of the progeny.

Variance component and narrow-sense heritability estimates for the two germination traits are given in Table 3. For percent germination of the interspecific hybrids, female general combining ability (GCA) variance was much larger than the male GCA variance for this trait, accounting for 20 and two percent of the total observed variation, respectively. This difference in additive variance estimates for the two species is also reflected in the much larger narrow-sense heritability estimate for Engelmann spruce--the female parent for all hybrid progeny. Specific combining ability (SCA) variance accounted for 15 percent of the total variation in percent germination.

For percent ungerminated-but-full seed among the full-sib interspecific families, both female and male GCA variance estimates were very small, accounting for only two and one percent of the total variation observed in this trait, respectively (Table 3). The small narrow-sense heritability estimates reflect the relatively small influence additive sources of variation have on percent

Table 2. Mean values of percent germination (% Germ) and percent ungerminated-but-full (% Ungf) seed on a total seed basis for open-pollinated (Open) and control-pollinated (Biparental and Selfed) collections.

Species	Open		Biparental		Selfed	
	%Germ	%Ungf	%Germ	%Ungf	%Germ	%Ungf
Blue spruce	43.1	4.9	11.9	2.2	4.8	1.0
Engelmann spruce	48.7	8.0	19.7	3.6	9.5	2.3
Engelmann x blue hybrids			0.30	0.46		

Table 3. Variance component and narrow-sense heritability estimates for production of viable and abnormal full-sib seed in Engelmann x blue spruce hybrids. Numbers in parentheses represent percentages of the respective variance components as compared to the total observed variance for that trait.

Trait[a]	Variance Component				Female h^2	Male h^2
	Female GCA	Male GCA	SCA	Error		
% Germ	0.079 (20%)	0.007 (2%)	0.061 (15%)	0.254 (63%)	0.80	0.09
% Ungf	0.044 (2%)	0.032 (1%)	0.464 (19%)	1.892 (78%)	0.07	0.05

[a] % Germ = percent germinated (viable) seed; % Ungf = percent ungerminated-but-full (abnormal) seed.

ungerminated-but-full hybrid seed. SCA varaince accounted for 19 percent of the observed variation in percent ungerminated-but-full seed.

Of the 158 hybrid germinants, 95 survived (60 percent), and isozymes of dormant vegetative buds from these seedlings were analyzed electrophoretically. Refer to Ernst et al. (1985a) for zymograms and inheritance data of the 11 loci analyzed in this study. Two loci--aldolase (ALD) and malate dehydrogenase(2) (MDH(2))--were monomorphic among all parents and their hybrid progeny. Glutamate oxaloacetate transaminase--(GOT(3))--was oppositely fixed among the blue and Engelmann spruce parents; the GOT(3)-1 allele in Engelmann spruce and GOT(3)-2 in blue spruce. Phosphoglucose isomerase--PGI(2)--was fixed among the Engelmann spruce parents for an allele not found in blue spruce--PGI(2)-1--and the blue spruce parents were either homozygous or heterozygous for alleles not found in Engelmann spruce--PGI(2)-3 and -4. Therefore, for both GOT(3)and PGI(2), hybrids exhibited heterozygous phenotypes not observed among the parents--GOT(3)-1/2, and PGI(2)-1/3 or PGI(2)-1/4.

Enough isozymic variation and parental combinations existed at the remaining seven loci to perform segregation tests on distributions of progeny genotypes, and the results are given in Table 4. No significant deviation was observed among cross-types--parental genotype combinations--of three loci--aconitase (ACO), glutamate

Table 4. Single-locus segregation tests of isozyme genotypes expressed in bud tissue of Engelmann x blue spruce full-sib progeny.

Enzyme (locus)	Cross type[a]	No. crosses	No. progeny	Progeny genotypes--observed(expected) 1/1	2/2	1/2	1/3	2/3	G[b]
ACO	1/1x1/2	8	39	23(19.5)		16(19.5)			1.26(1)
IDH(2)	1/2x2/2	4	7		7(3.5)	0(3.5)			9.70(1)
6PG(1)	1/1x1/3	4	17	13(8.5)			4(8.5)		5.02(1)
	1/1x1/2	6	13	12(6.5)		1(6.5)			10.97(1)
	1/2x1/3	2	18	9(4.5)		1(4.5)	4(4.5)	4(4.5)	7.58(3)
	1/2x3/3	1	1				------	1(---)	------
GDH	1/2x2/2	4	12		9(6)	3(6)			3.14(1)
PGM(1)	1/2x2/2	12	65		41(32.5)	24(32.5)			4.50(1)
MDH(3)	1/1x1/2	2	7	4(3.5)		3(3.5)			0.14(1)
	1/2x1/2	3	15	7(3.75)	4(3.75)	4(7.5)			4.23(2)
	1/2x2/2	6	34		24(17)	10(17)			5.94(1)
MDH(4)	1/2x2/2	6	29		17(14.5)	12(14.5)			0.87(1)
	1/2x2/3	2	6		3(1.5)	1(1.5)	1(1.5)	1(1.5)	1.73(3)
	1/2x3/3	1	6				4(3)	2(3)	0.68(1)
	2/2x2/3	5	7		3(3.5)			4(3.5)	0.14(1)

[a] Progeny resulting from crosses between homozygous parents always bred true and therefore are not shown.
[b] G values corresponding to various significance levels and degrees of freedom are:
0.05 significance level: 3.84 (1df), 5.99 (2df) and 7.81 (3df);
0.01 significance level: 6.63 (1df), 9.21 (2df) and 11.34 (3df).

dehydrogenase (GDH) and malate dehydrogenase(4) (MDH(4)). Only one cross-type--1/2 x 2/2--deviated signifanctly for MDH(3), and that was the result of a large deviation in observed versus expected progeny genotypes in a single full-sib hybrid family. Tests of the other two cross-types for MDH(3) did not indicate any deviation from expected. The progeny distributions for phosphoglucomutase--PGM(1)--and isocitrate dehydrogenase--IDH(2)--deviated significantly at the 0.05 and 0.01 levels, respectively. Among the three cross-types for 6-phosphogluconate dehydrogenase--6PG(1)--progeny genotype distributions deviated significantly for two of them. Therefore there is some evidence for gametic selection or hybrid inviability among the full-sib hybrid progeny based on isozyme genotypes at three or four of the 11 loci. Unique hybrid allozyme phenotypes--i.e., banding patterns not observed among the intraspecific progeny (see Ernst et al. 1985a)--observed among the hybrid progeny are shown in Figure 1.

From the zone 3 Scotch Creek population, the putative hybrid swarm area, 245 embryos from nine half-sib blue spruce families and 357 embryos from 11 half-sib Engelmann spruce families were analzyed electrophoretically. No interspecific hybrids were observed among the embryos based on electrophoretic phenotypes.

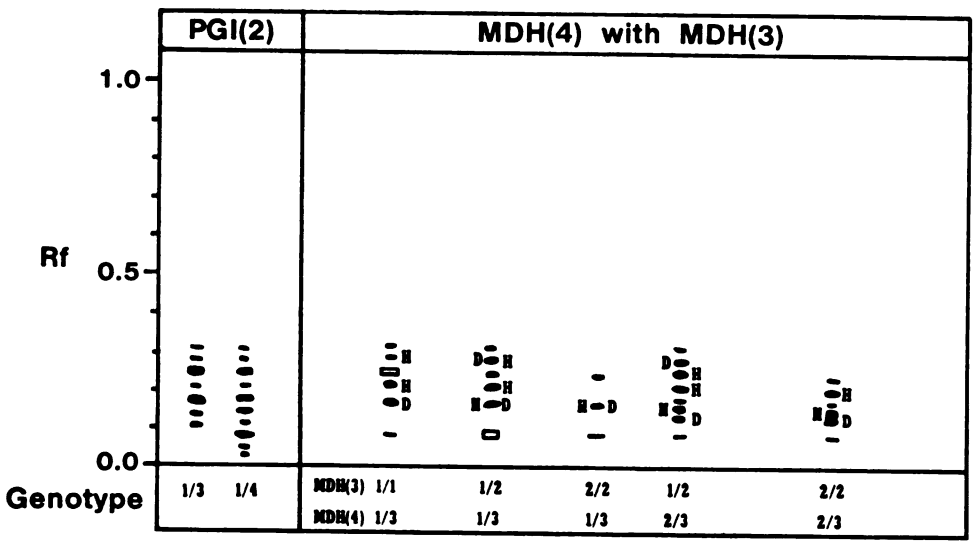


Figure 1. Unique isozyme phenotypes expressed in bud tissue of Engelmann x blue spruce full-sib progeny but not among intraspecific progeny. Heterodimers are marked as 'H' and homodimers as 'D', and null allozymes are represented by empty boxes.

DISCUSSION

Interspecific hybrids between blue and Engelmann spruce were positively identifiable using isozyme analysis. In a related study in the Dolores River drainage, the two species exhibited several allelic differences among the 13 loci sampled (Ernst et al. 1985b), and genotypes of known hybrids in this study were consistent with parental genotypes across the 11 loci assayed. The locus best suited for identification of interspecific hybrids in the Dolores River drainage was PGI(2). Allele 1 was observed only in Engelmann spruce--frequency >0.99--while alleles 3 and 4 were observed only in blue spruce--frequencies of >0.95 and <0.01, respectively (Ernst et al. 1985b). Allele 2 was observed in both species, but at frequencies of less than 0.01 in Engelmann spruce and 0.04 in blue spruce. Therefore, any individuals in the Dolores River drainage which are heterozygous as PGI(2)-1/3 or -1/4 are very strong candidates for interspecific hybrids. In combination with genotypes at other loci which differ in allelic frequency or composition between the two species--GOT(3), IDH(2), 6PG(1), GDH, PGM(1), MDH(4), acid phosphatase (ACP(2)), and diaphorase (DIA(2)) (Ernst et al. 1985b)--hybrids can be readily confirmed. How well these species differences are maintained in other portions of the ranges of blue and Engelmann spruce must await further study.

Progeny genotypic distributions for at least three of the 11 isozyme loci assayed in this study deviated from the expected values, suggesting some selection may occur at gametic or embryonic stages. This is not surprising based on the hybrid inviability--abnormalities and poor survival of hybrids--observed in this study and also in the study by Fechner and Clark (1969). It is interesting to note that for all loci where progeny distributions did deviate from the expected, the imbalance was always towards the allele more common to both species--IDH(2)-2, 6PG(1)-1 and PGM(1)-2 (Table 2)--rather than the allele unique or more common to only one species (see also Ernst et al. 1985b).

Based on the results from reciprocal interspecific hybridizations among the 20 blue spruce and 20 Engelmann spruce parents in this study, hybridization between the two species is unidirectional and of very low crossability. Viable seed was obtained only with Engelmann spruce as the female parent, with an average of 0.30 percent germinated seed on a total seed basis across all 20 Engelmann spruce females. These results are similar to those obtained by Fechner and Clark (1969), although their controlled crosses were limited to one female parent of each species and two blue spruce pollen parents and one Engelmann spruce pollen parent. They reported viable seed only with Engelmann spruce as the female parent and very low interspecific crossability--mean viable seed yields of less than two

percent. Fechner and Clark (1969) also reported a high frequency of hybrid abnormalities, subh as reverse germination, termination of germination after emergence of the radicle, and branched hypocotyls. However, they did not report multiple embryony, which was very prevalent among the viable hybrid seedlots of the present study. Archegonia with multiple nuclei the size of the egg were reported in blue spruce ovules pollinated with Engelmann spruce pollen (Kossuth and Fechner 1973).

In an anatomical study of ovule development among reciprocal interspecific crosses between blue and Engelmann spruce, Kossuth and Fechner (1973) reported no viable seed for the Engelmann x blue (female x male) spruce cross and 0.48 percent germination for the blue x Engelmann spruce cross. They also used a limited number of parents, with one female of each species and a two-tree mix of blue spruce pollen and one Engelmann spruce pollen parent. They did not observe any pollen tubes penetrating the nucellus among the Engelmann x blue spruce ovules. In the reciprocal cross, most pollen also died or pollen tubes did not grow rapidly, but some pollen tubes did penetrate the nucellus and archegonium, dead hybrid embryos were observed, and a few viable seed were obtained. Kossuth and Fechner (1973) also observed that incompatibility breakdowns occurred primarily between nine and 30 days after pollination and before fertilization, as evidenced by termination of female gametophyte development and necrosis.

They attributed this breakdown to a lack of intraspecific pollen rather than a result of incompatible pollen because normal female gametophyte development is a response to the presence of intraspecific pollen, even if ungerminated (see also Mikkola 1969).

The results presented by Kossuth and Fechner (1973) indicate the interspecific cross between blue and Engelmann spruce should also be possible with blue spruce as the female parent--i.e., it is bidirectional. This contrasts strongly with the results of this study, as none of the 20 blue spruce females crossed successfully with Engelmann spruce--a total of 60 blue x Engelmann full-sib families. The parents were located in areas where both species were present at least to a limited degree, and cross-compatibility may not be so restricted among allopatric populations of the two species. This could be easily tested. This phenomenon has been documented in Drosophila paulistorum, where incompatibility is stronger among sympatric populations of several subspecies of D. paulistorum than among allopatric populations of the same subspecies (Ayala et al. 1974; Ehrman 1965). A similar situation was also observed in Gilia, where sympatric species exhibited stronger incompatibility than did allopatric species of this same genus (Grant 1966).

The results from a related study of isozyme variation among the blue and Engelmann spruce populations in the

Dolores River drainage suggests the elevationally allopatric blue and Engelmann spruce subpopulations in this drainage may be less divergent genetically than the sympatric subpopulations (Ernst et al. 1985b). This may indicate there is some selectin against interspecific hybridization in the sympatric zone, and cross-compatibility may be less restricted among the allolpatric subpopulations. It may also be a function of the habitats the subpopulations occupy and the extent of variability within each of the subpopulations. In the studies by Daubenmire (1972) and Taylor et al. (1975), less variation was observed in morphological and phenolic characters in the sympatric populations than in the allopatric populations. Also, using a discriminant function composed from morphological and terpenoid characters, Schaefer and Hanover (1985c) found evidence of introgression among the zone 3 Scotch Creek subpopulations of blue and Engelmann spruce, while the zone 1 and zone 5 "pure species" subpopulations were readily separable by the same discriminant function. The putative hybrids they identified resembled Engelmann spruce more strongly than blue spruce, suggesting gene flow is favored towards Engelmann spruce rather than blue spruce.

Differential cross-compatibility between sympatric and allopatric populations of blue and Engelmann spruce may exist and the interspecific cross may be bidirectional. However, the unidirectional crossability reported by

Fechner and Clark (1969) and in this study, and evidence for unidirectional gene flow in morphological and terpenoid characters (Schaefer and Hanover 1985c) suggest the cross is compatible only with Engelmann spruce as the female parent. The results of the study by Kossuth and Fechner (1973) indicate the reciprocal cross may be feasible as well. The many instances of gametophytic breakdown and irregularities in the archegonia in conjunction with low seed set support their conclusion. It is also possible the hybrids obtained by Kossuth and Fechner (1973) were intraspecific contaminants. The hybridity of the progeny could not be confirmed because they were accidentally destroyed (Fechner, personal communication). Four intraspecific contaminanted seedlings were observed in three different blue x Engelmann spruce family replicates in this study and were documented as such only by electrophoretic analysis. The reduced viable seed yields--low percent germination--of these families suggested the four seedlings were interspecific hybrids, but they did not possess isozyme genotypes characteristic of the respective parents. The seedlings apparently resulted from very slight pollen contamination, and because of the strong hybridization barriers between blue and Engelmann spruce the few blue spruce pollen grains that were present in these bags were manifest.

In the Engelmann x blue spruce cross, both additive and nonadditive sources of genetic variation appear to

exert an influence in the production of viable hybrid seed. Additive sources of variation in the female parent, Engelmann spruce, are apparently very important in the production of viable interspecific seed while they are not in the male parent, blue spruce. Maternal effects may be confounded in the large female additive variance estimate. The maternal effects varaince could not be separated because it could not be assumed the additive genetic variance was equivalent in both species. However, maternal effects were not present in intraspecific crosses of Engelmann spruce (Ernst et al. 1985). Certain Engelmann spruce females crossed much better than others with a variety of blue spruce pollen parents; e.g., six Engelmann spruce females produced viable hybrid seed, and in greater amounts, with all three blue spruce pollen parents they were crossed to, while two Engelmann spruce female parents produced viable seed in crosses with only two blue spruce males, eight Engelmann spruce females crossed with only one blue spruce male, and four Engelmann spruce females did not produce any viable hybrid seed. Whether these differences are truly genetic or environmental--maternal--in origin must await further testing. The influlence of maternal effects in the Engelmann x blue spruce cross can be tested by replicating the same crosses in seed orchards of equivalent genotypic composition located at two or more sites. The influence of nonadditive sources of variation

is supported by the fact that certain full-sib families produced much larger quantities of hybrid seed--three to six times the overall mean.

No mature F_1 hybrids were observed in the Dolores River drainage based on morphological and isozymic phenotypes of 56 blue spruce and 76 Engelmann spruce individuals (see also Ernst et al. 1985b). The sampled individuals included the 40 parents used to make the interspecific crosses in this study. One blue spruce individual from the lower elevation, pure blue spruce zone of the Dolores River was heterozygous for GOT(3)-1/2 (see Ernst et al. 1985b). However, this individual possessed isozyme genotypes characteristic of blue spruce at all other loci and resembled blue spruce morphologically. Therefore, based on the location of the individual and its resemblance to blue spruce morphologically and in isozymic composition, the presence of GOT(3)-1 in blue spruce probably represents a rare allele in this species rather than a result of interspecific gene flow (introgression).

Because of the very low crossability and extent of hybrid inviability observed in this and previous studies (Fechner and Clark 1969; Kossuth and Fechner 1973) under the best of conditions--artificial pollination with no intraspecific pollen competition and a controlled germination environment--it was not expected that mature F_1 hybrids would be found. Based on isozyme genotypes there was no evidence of introgression among these individuals or

their progeny either. Therefore, if natural hybrids do exist, they must be very rare--one tree in several million at best--and introgression very localized and "diluted" if the hybrids are indeed fertile. The hybrids produced artificially in this study will be grown to maturity to determine if they are fertile and can be backcrossed successfully to the two pure species parents.

No interspecific F_1 hybrids between blue and Engelmann spruce were observed when 602 open-pollinated embryos from single-tree cone collections of nine blue spruce and 11 Engelmann spruce parents in zone 3 of Scotch Creek were analyzed electrophoretically. Embryos from seed of blue and Engelmann spruce trees in zone 3 of Scotch Creek were assayed because there was a much greater probability for natural interspecific pollination at that site, and the embryos had not been subjected to the environment of germination and seedling establishment in the field. However, tens of thousands of embryos must be screened in the hopes of finding a natural hybrid embryo because of intraspecific pollen competition and low interspecific crossability. The screening of embryos in this study represents a small fraction of the required sample size. It may be easier to analyze electrophoretically only those embryos which show abnormal germination characteristics similar to those exrpessed by the hybrids produced artificially in this study.

The concept of low and unidirectional crossability between blue and Engelmann spruce is consistent with both species having maintained their species identities with little if any evidence of natural hybridization and introgression. Because air flow in the mountain valleys occupied by the two species is generally from high to low elevation, the predominant direction of pollen flow will be from Engelmann spruce to blue spruce. For the two species to maintain their species identities, selection pressure is predominately upon blue spruce as the female parent because blue spruce is more likely to be in the presence of Engelmann spruce pollen than the reverse scenario. Therefore, while crossability is expected to be very low between blue and Engelmann spruce, genetic barriers to hybridization may be even stronger--or complete--with blue spruce as the female parent.

CHAPTER VI

RECOMMENDATIONS FOR FUTURE STUDY

Based on the results of this study, a variety of research directions can be pursued to further assess the degree of natural hybridization and introgression between blue and Engelmann spruce. The following represents a partial list of such studies as suggested by this author. No attempt was made to prioritize the suggestions.

1. Determine the extent of maternal effects in blue spruce. This can be accomplished by replicating given crosses in seedling or clonal seed orchards over a variety of sites.
2. Determine the extent of isozyme variability in blue, Engelmann and white spruce throughout their respective ranges. This would be best accomplished by sampling as many individuals as possible (e.g., 50 single-tree collections) from a variety of locations throughout the range of each species.
3. Increase the number of enzyme systems which can be assayed in blue and Engelmann spruce, and determine the inheritance of the isozymes in each enzyme system.
4. Conduct further interspecific crosses between blue and Engelmann spruce to determine if the cross is truly reciprocal as suggested by Kossuth and Fechner (1973). These crosses should include parents of blue and Engelmann spruce from areas of allopatry.

5. Conduct studies in other unique populations of blue, Engelmann and white spruce; e.g., outlying populations in New Mexico, Arizona, Wyoming and Montana, as well as locations where both blue and Engelmann spruce (and possibly white spruce) occur in sympatry and cross-pollination is probable.
6. Compare morphological, anatomical, biochemical and physiological traits of blue and Engelmann spruce when grown in common garden experiments on a variety of sites. A majority of the studies comparing such traits in blue and Engelmann spruce have sampled from individuals in situ, and environmental influences greatly interfere in making accurate genetic comparisons.
7. Investigate the physiological and genetic basis for incompatibility between blue and Engelmann spruce. Essentially no studies have been conducted on compatibility/incompatibility mechanisms in gymnosperms, yet these 'naked seed' plants offer the simplest of conditions because there no intermediary tissues between the pollen grain and ovule. Such an understanding may also shed light on the mode of speciation in blue and Engelmann spruce.
8. Compare the viability and growth, morphology, anatomy, biochemistry, physiology, cytogenetics, fertility and crossability of the F_1 Engelmann x blue spruce hybrids

generated in this study relative to the pure species. The question of hybrid viability and fertility is of utmost importance in evaluating possible modes of speciation and adaptation in blue and Engelmann spruce.

9. Incorporate best linear unbiased prediction (BLUP) techniques into the breeding evaluation procedures of forest trees and other plant species. BLUP and associated selection index techniques allow unprecedented flexibility for unbalanced data situations, a common occurrence in plant breeding experiments.

LIST OF REFERENCES

LIST OF REFERENCES

Adams, W.T., and S. Coutinho. 1977. Isozyme genetic markers useful for studies of the Pinus rigida x Pinus taeda hybrid. New Hampshire Agri. Expt. Sta. Scient. Cntr. No. 847.

Adams, W.T., and R.J. Joly. 1980. Genetics of allozyme variants is loblolly pine. J. Hered. 71: 33-40.

Allen, G.S., and J.N. Owens. 1972. The life history of Douglas-fir. Cat. No. Fo42-4972, Information Canada, Ottawa.

Anand, I.J., and B.R. Murty. 1969. Serial analysis of combining ability in diallel and fractional diallel crosses in linseed. Theor. Appl. Genet. 39: 88-94.

Ayala, F.J. 1975. Genetic differentiation during the speciation process. Evol. Biol. 8: 1-78.

_____. 1982. Population and evolutionary genetics: a primer. Benjamin/Cummings Publ. Co., Inc., Menlo Park, CA.

_____, M.L. Tracey, D. Hedgecock and R.C. Richmond. 1974. Genetic differentiation during the speciation process in Drosophila. Evolution 28: 576-592.

Banks, B.D., I.L. Mao and J.P. Walter. 1985. Robustness of the restricted maximum likelihood estimator derived under normality as applied to data with skewed distributions. J. Dairy Sci. 68: 1785-1792.

Bonga, J.M. 1981. Vegetative propagation of mature trees by tissue culture. p. 191-196 In A.N. Rao (ed.), Proc. COSTED Symp. on Tissue Culture of Economically Important Plants. Singapore, 1981.

Bramlett, D.L., T.R. Dell and W.D. Pepper. 1983. Genetic and maternal influences on Virginia pine seed germination. Silvae Genet. 32: 1-4.

Cecich, R.A. 1979. Ovule development and abortion in Pinus banksiana. p. 33-40 In Proc. Joint IUFRO Symp. on Flowering and Seed Development in Trees, May 15-18, 1978, Mississippi State Univ., Starkville, MS.

Chaudhary, B.D., S.N. Kakar and R.K. Singh. 1977. Estimation of genetic parameters in barley (Hordeum vulgare L.). II. Partial diallel analysis. Theor. Appl. Genet. 49: 153-156.

Cheliak, W.M., and J.A. Pitel. 1984. Genetic control of allozyme variants in mature tissues of white spruce trees. J. Hered. 75: 34-40.

Clayton, J.W., and D.N. Tretiak. 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. J. Fish. Res. Board Can. 29: 1169-1172.

Cram, W.H. 1984. Some effects of self-, cross-, and open-pollinations in Picea pungens. Can. J. Bot. 62: 392-395.

Daubenmire, R. 1972. On the relation between Picea pungens and Picea engelmannii in the Rocky Mountains. Can. J. Bot. 50: 733-742.

Dowling, T.E., and W.S. Moore. 1984. A program for estimating genetic variability within and between populations. J. Hered. 75: 34-40.

Eckert, R.T., R.L. Joly, and D.B. Neale. 1981. Genetics of isozyme variants and linkage relationships among allozyme loci in 35 eastern white pine clones. Can. J. For. Res. 11: 573-579.

Ehrman, L. 1965. Direct observation of sexual isolation between allopatric and between sympatric strains of the different Drosophila paulistorum races. Evolution 19: 459-464.

El-Kassaby, Y.A. 1981. Genetic interpretation of malate dehydrogenase isozymes in some conifer species. J. Hered. 72: 451-452.

_____, F.C. Yeh and O. Sziklai. 1981. Inheritance of allozyme variants in coastal Douglas-fir (Pseudotsuga menziesii var. menziesii). Can. J. Genet. Cytol. 24: 325-335.

Ernst, S.G., J.W. Hanover and D.E. Keathley. 1985a. Inheritance of isozymes in seed and bud tissues of blue and Engelmann spruce. In preparation.

_____. 1985b. Allozyme variation of blue and Engelmann spruce in southwestern Colorado. In preparation.

_____. 1985c. Assessment of natural hybridization and introgression of blue and Engelmann spruce. In preparation.

_____, and I.L. Mao. 1985. Genetic variation and control of intraspecific crossability in blue and Engelmann spruce. In preparation.

Falconer, D.S. 1960. Introduction to quantitative genetics. Ronald Press, New York.

Fechner, G.H. 1979. The biology of flowering and fertilization. p. 1-24 In Proc. Joint IUFRO Symp. on Flowering and Seed Development in Trees, May 15-18, 1978, Mississippi State Univ., Starkville, MS.

_____, and R.W. Clark. 1969. Preliminary observations on hybridization of Rocky Mountain spruces. In Proc. of the Comm. on Forest Tree Breeding in Canada 11: 237-247.

Finnerty, V., and G. Johnson. 1979. Post-translational modification as a potential explanation of high levels of enzyme polymorphism: xanthine dehydrogenase and aldehyde oxidase in Drosophila melanogaster. Genetics 91: 695-722.

Fowler, D.P., and L. Roche. 1975. Genetics of Engelmann spruce. USDA For. Serv. Res. Pap. WO-30.

Franklin, E.C. 1970. Survey of mutant forms and inbreeding depression in species of the family Pinaceae. USDA For. Serv. Res. Pap. SE-61.

Futuyma, D.J. 1979. Evolutionary biology. Sinauer Assoc., Inc., Sunderland, MA.

Gordon, G.H. 1980. A method of parental selection and cross prediction using incomplete partial diallels. Part I: a simulation study. Theor. Appl. Genet. 56: 225-232.

Grant, V. 1966. The selective origin of incompatibility barriers in the plant genus Gilia. Amer. Nat. 100: 99-118.

_____. 1981. Plant speciation (Second edition). Columbia Univ. Press, New York.

Greathouse, T.E. 1966. Inheritance of germinative energy and germinative capacity in Douglas-fir. p. 60-62 In Joint Proc. Second Genetics Workshop Soc. Am. For. and Seventh Lake States For. Tree Improv. Conf., USDA For. Serv. Res. Pap. NC-6.

Gupta, D., and S.L. Basak. 1983. Genetics of germination and seedling growth of flax (Linum usitatissimum). Seed Sci. and Technol. 11: 251-256.

Guries, R.P., and F.T. Ledig. 1978. Inheritance of some polymorphic isoenzymes in pitch pine (Pinus rigida Mill.). Heredity 40: 27-32.

_____. 1981. Genetic diversity and population structure in pitch pine (Pinus rigida Mill.). Evolution 36: 387-402.

Habeck, J.R., and T.W. Weaver. 1969. A chemosystematic analysis of some hybrid spruce (Picea) populations in Montana. Can. J. Bot. 47: 1565-1570.

Hallauer, A.R. 1981. Selection and breeding methods. p. 3-55 In K.J. Frey (ed.), Plant Breeding II, Iowa State Univ. Press, Ames, IA.

Hamrick, J.L., J.B. Mitton and Y.B. Linhart. 1981. Levels of genetic variation in trees: influence of life history characteristics. p. 35-41 In Proc. of the Symp. on Isozymes of North Amer. Forest Trees and Forest Insects, USDA For. Serv. Gen. Tech. Rep. PSW-48.

Hanover, J.W. 1975. Genetics of blue spruce. USDA For. Serv. Res. Pap. WO-28.

_____, E. Young, W.A. Lemmien and M. Van Slooten. 1976. Accelerated-Optimal-Growth: a new concept in tree production. Michigan State Univ. Agric. Exper. Sta. Res. Pap. No. 317.

Harris, H., and D.A. Hopkinson. 1972. Average heterozygosity in man. Ann. Human Genet. 36: 9-20.

Heit, C.E. 1961. Laboratory germination and recommended testing methods for 16 spruce (Picea) species. Proc. of the Assoc. of Official Seed Analysts of North Amer. 51: 165-171.

Henderson, C.R. 1973. Sire evaluation and genetic trends. p. 10 In Proc. of the Animal Breeding and Genetics Symposium in honor of Dr. J.L. Lush, ASAS, ADSA, Champaign, IL.

Jones, J.R., and N.T. Bernard. 1977. How to tell Engelmann from blue spruce in the Southwest. USDA For. Serv. Gen. Tech. Rep. RM-34.

Kearsey, M.J. 1965. Biometrical analysis of a random mating population: a comparison of five experimental designs. Heredity 20: 205-235.

Kempthorne, O., and R.N. Curnow. 1961. The partial diallel cross. Biometrics 17: 229-250.

King, J.P., P.O. Rudolf, R.M. Jeffers and H. Nienstaedt. 1970. Effects of varying proportions of self-pollen in seed yield, seed quality and seedling development in Picea glauca. Paper presented and distributed at the Meeting of the Working Group in Sexual Reproduction of Forest Trees, IUFRO Sec. 22, Varparanta, Finland, May, 1970.

Kossuth, S.V., and G.H. Fechner. 1973. Incompatibility between Picea pungens Engelm. and Picea engelmannii Parry. Forest Sci. 19: 50-60.

Kudray, G.M., and J.W. Hanover. 1980. A preliminary evaluation of the Spartan spruce (Picea glauca x P. pungens). Michigan State Univ. Agri. Expt. Sta. Res. Pap. 405.

Leigh Brown, A.J., and C.H. Langley. 1979. Reevaluation of level of genic heterozygosity in natural populations of Drosophila melanogaster by two-dimensional electrophoresis. Proc. Natl. Acad. Sci. USA 76: 2381-2384.

Lewontin, R.C. 1974. The genetic basis of evolutionary change. Columbia Univ. Press, New York.

Lundkvist, K. 1975. Inheritance of acid phosphatase isozymes in Picea abies. Hereditas 79: 221-226.

Mao, I.L. 1982. Modeling and data analysis in animal breeding: notes for an internordic post-graduate course. Dept. of Animal Breeding and Genetics, Sweedish Univ. of Agric. Sci., Uppsala, Sweden.

Mayr, E. 1963. Animal species and evolution. Harvard Univ. Press, Cambridge, MA.

Millar, C.I. 1983. A steep cline in Pinus muricata. Evolution 37: 311-319.

Mitton, J.B., and R. Andalora. 1981. Genetic and morphological relationships between blue spruce, Picea pungens Engelm. and Engelmann spruce, Picea engelmannii Parry in the Colorado Front Range. Can. J. Bot. 59: 2088-2094.

Mitton, J.B., Y.B. Linhart, J.L. Hamrick and J.S. Beckman. 1977. Observations on the genetic structure and mating system of ponderosa pine in the Colorado Front Range. Theor. Appl. Genet. 51: 5-13.

Mitton, J.B., Y.B. Linhart, K.B. Sturgeon and J.L. Hamrick. 1979. Allozyme polymorphisms detected in mature needle tissue of ponderosa pine. J. Hered. 70: 86-89.

Morgenstern, E.K. 1974. A diallel cross in black spruce, Picea mariana (Mill.) B.S.P. Silvae Genet. 23: 67-70.

Morris, R.W., and P.T. Spieth. 1978. Sampling strategies for using female gametophytes to estimate heterozygosity in conifers. Theor. Appl. Genet. 51: 217-222.

Muller, G. 1977. Short note: cross-fertilization in a conifer stand inferred from enzyme gene-markers in seeds. Silvae Genet. 26: 223-226.

Murty, B.R., V. Arunachalam and I.J. Anand. 1967. Diallel and partial diallel analysis of some yield factors in Linum usitatissimum. Heredity 22: 35-41.

Namkoong, G., and J.H. Roberds. 1974. Choosing mating designs to efficiently estimate genetic variance components for trees. Silvae Genet. 23: 43-53.

Neale, D.B., and W.T. Adams. 1981. Inheritance of isozyme variants in seed tissues of balsam fir (Abies balsamea). Can. J. Bot. 59: 1285-1291.

Neale, D.B., J.C. Weber and W.T. Adams. 1984. Inheritance of needle tissue isozymes in Douglas-fir. Can. J. Genet. Cytol. 26: 459-468.

Nei, M. 1972. Genetic distance between populations. Amer. Nat. 106: 283-292.

_____. 1977. F-statistics and analysis of gene diversity in subdivided populations. Ann. Human Genet. 41: 225-233.

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

_____, and A.K. Roychoudhury. 1974. Sampling varianices of heterozygosity and genetic distance. Genetics 76: 379-390.

Newton, K.J. 1979. A gene which alters the electrophoretic mobilities of maize mitochondrial malate dehydrogenase isozymes (Abstract). Genetics 91 (Suppl.): s88-s89.

Nguyen, H.T., and D.A. Sleper. 1983. Genetic variability of seed yield and reproductive characters in tall fescue. Crop Sci. 23: 621-626.

Nienstaedt, H., and A. Teich. 1971. The genetics of white spruce. USDA For. Serv. Res. Pap. WO-15.

O'Malley, D.M., F.W. Allendorf and G.M. Blake. 1979. Inheritance of isozyme variation and heterozygosity in Pinus ponderosa. Biochem. Genet. 17: 233-250.

O'Malley, D.M., N.C. Wheeler and R.P. Guries. 1980. A manual for starch gel electrophoresis. Univ. of Wisconsin Dept. of Forestry Staff Pap. No. 11.

Pederson, D.G. 1972. A comparison of four experimental designs for the estimation of heritability. Theor. Appl. Genet. 42: 371-377.

Pryor, A.J. 1974. Allelic glutamic dehydrogenase isozymes in maize--a single hybrid isozyme in heterozygotes? Heredity 32: 397-401.

Reed, A.N.F., and J.W. Hanover. 1983. Geographic variation in cortical monoterpenes of blue spruce, Picea pungens. Paper presented at the 7th North American Forest Biology Workshop, 1982, Univ. of Kentucky, Louisville.

Ridgway, G.J., S.W. Sherburne and R.D. Lewis. 1970. Polymorphisms in the esterases of Atlantic herring. Trans. Am. Fisheries Soc. 99: 147-151.

Rogers, J.S. 1972. Measures of genetic similarity and genetic distance. Studies in Genetics VII. Univ. Texas Publ. 7213 145-153.

Rudin, D., and I. Ekberg. 1978. Linkage studies in Pinus sylvestris L. using macro gametophyte allozymes. Silvae Genet. 27: 1-12.

Sarich, V.M. 1977. Rates, sample sizes, and the neutrality hypothesis for electrophoresis in evolutionary studies. Nature 265: 24-28.

Scandalios, J.G. 1969. Genetic control of multiple molecular forms of enzymes in plants: a review. Biochem. Genet. 3: 37-79.

Schaefer, P.R., and J.W. Hanover. 1985a. A morphological comparison of blue and Engelmann spruce in southwestern Colorado. Silvae Genet. (in press).

_____. 1985b. Monoterpene concentrations in cortical oleoresin of blue and Engelmann spruce. Forest Sci. (in press).

_____. 1985c. Evidence of natural hybridization between blue and Engelmann spruce in southwestern Colorado. In preparation.

Schaeffer, L.R. 1976. Maximum likelihood estimation of variance components in dairy cattle breeding research. J. Dairy Sci. 59: 2146-2151.

Shaw, D.V., and R.W. Allard. 1981. Analysis of mating system parameters and population structure in Douglas-fir using single-locus and multilocus methods. p. 18-22 In Proc. of the Symp. on Isozymes of North American Forest Trees and Forest Insects. USDA For. Serv. Gen. Tech. Rep. PSW-48.

Slaughter, C.A., D.A. Hopkinson and H. Harris. 1975. Aconitase polymorphism in man. Ann. Hum. Genet. 39: 193-202.

Sokal, R.R., and F.J. Rohlf. 1969. Biometry. W.H. Freeman, San Francisco.

Taylor, R.J., S. Williams and R. Daubenmire. 1975. Interspecific relatinships and the question of introgression between Picea engelmannii and Picea pungens. Can. J. Bot. 53: 2547-2555.

Tigerstedt, P.M.A. 1974. The application of ecological genetics principles to forest tree breeding. Silvae Genet. 23: 62-66.

Timmis, R., and G.A. Ritchie. 1984. Progress in Douglas-fir tissue culture. p. 37-46 In Proc. Intl. Symp. of Recent Advances in Forest Biotechnology, Michigan Biotechnology Institute, Michigan State Univ., East Lansing, MI.

Wehner, T.C. 1984. Estimates of heritabilities and variance components for low-temperature germination ability in cucumber. J. Amer. Soc. Hort. Sci. 109: 664-667.

Wendel, J.F., and C.R. Parks. 1982. Genetic control of isozyme variation in Camelia japonica L. J. Hered. 73: 197-204.

Wheeler, N.C., and R.P. Guries. 1982. Population structure, genic diversity, and morphological variation in Pinus contorta Dougl. Can. J. For. Res. 12: 595-606.

Wright, J.W. 1955. Species crossability in spruce in relation to distribution and taxonomy. Forest Sci. 1: 319-349.

_____. 1976. Introduction to forest genetics. Academic Press, New York.

Wright, S. 1951. The genetical structure of populations. Ann. Eugen. 15: 323-354.

_____. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution 19: 395-420.

Yeh, F.C., and Y.A. El-Kassaby. 1979. Enzyme variations in natural populations of Sitka spruce (Picea sitchensis (Bong.) Carr.). I. Genetic variation patterns in ten IUFRO provenances. p. 25 In Proc. 52nd Northwest Sci. Assoc. Meeting.

Yeh, F.C., and D. O'Malley. 1980. Enzyme variations in natural populations of Douglas-fir, Pseudotsuga menziesii (Mirb.) Franco, from British Columbia. I. Genetic variation patterns in coastal populations. Silvae Genet. 29: 83-92.

Zouros, E. 1976. Hybrid molecules and the superiority of the heterozygote. Nature (London) 262: 227-229.