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MORPHOLOGICAL TRAIT ANALYSIS OF
SYNTHETIC PICEA ENGELMANNII Parry x
P. PUNGENS Engelm. HYBRIDS

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Karl F. Gruber

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MORPHOLOGICAL TRAIT ANALYSIS OF SYNTHETIC *PICEA ENGELMANNII* Parry x *P. PUNGENS* Engelm. HYBRIDS

By

Karl F. Gruber

A THESIS

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ABSTRACT

MORPHOLOGICAL TRAIT ANALYSIS OF SYNTHETIC HYBRIDS OF *PICEA ENGELMANNII* Parry x *P. PUNGENS* Engelm.

By

Karl F. Gruber

The species identity of individual trees was confirmed through isoenzyme electrophoresis examination of the phosphoglucoisomerase (PGI) locus in blue spruce, Engelmann spruce and artificial Engelmann spruce x blue spruce hybrids. Polymorphisms of the slower migrating isoenzyme, PGI-2, consistently distinguished the species and hybrids.

Four foliage traits which distinguish blue spruce from Engelmann spruce were measured in the above trees and subjected to analyses of variance, linear regression, and canonical variates discriminant function analysis. All group means were significantly different from each other for each trait, and the hybrid group was always quantitatively intermediate, yet there was a great degree of overlap between taxa. The measured traits were positively correlated with one another. Discriminant function analysis separated blue and Engelmann spruce, but failed to distinguish hybrids from pure species with certainty. Foliage color, height, diameter, and terminal bud length and width were also measured in the three study groups.

ACKNOWLEDGEMENTS

This work represents the fruit of my labor which was made possible through the patience, guidance and support of my professor, Dr. Jim Hanover. His wisdom has enlightened me in many ways, but his example of honesty and kindness in everyday life has been the greatest of lessons I have experienced in my graduate career. I also thank my committee members, Dr. Daniel Keathley and Dr. Jim Hancock, for their helpful criticisms and their patience. In addition, I extend my appreciation to Roy Prentice, Paul Bloese, and Ray Miller for all of their help and advice as well as to my fellow graduate students, my comrades.

Most of all, I thank my wife, Susan, for her love and companionship throughout this long endeavor, and our parents for their steadfast support.

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CHAPTER I

INTRODUCTION

The relationship between blue spruce (*Picea pungens* engelm.) and Engelmann spruce (*Picea engelmannii* Parry) has been extensively documented and researched in the past. Wright(1955) was the first to attempt to describe the genealogical relationship between these and other spruce species, and classified them as closely related within the white spruce complex of Western North America. Both species occupied a much larger range during the last ice age, stretching across the Great Plains of what is now the United States south to present day Mexico. Since the warming of the last 10,000 years, their range has dramatically shrunk to the montane habitat of the Rocky Mountains. The Dolores River and subtending Scotch Creek drainage in Southern Colorado is one such area where both blue spruce and Engelmann spruce flourish sympatrically.

It is in the Scotch Creek / Dolores River geographic vicinity that both blue and Engelmann spruce populations overlap; the blue spruce occupying drier sites, southern exposures, and lower elevations while Engelmann spruce occurs on moist, northern exposures and higher elevations. In the areas of sympatry, between 2670 and 2820 meters elevation, putative hybrid swarms occur.

These putative hybrids have been classified as such due to intermediacy of traits such as foliage size, shape and sharpness, branching habit and crown appearance, twig color and pubescence, and cone and bud shape characteristics, all of which have been used in the past to separate the two pure species(Jones and Bernard,1977, Taylor, Williams and Daubenmire, 1975, Daubenmire,1972, Mitton and Andalora, 1981, Schaefer and Hanover,1985,1987,1990). Cortical oleoresin monoterpene composition has also been cited as diagnostic in distinguishing pure species and identifying putative hybrids (Schaefer and Hanover,1987).

Ernst (1985) made controlled crosses of individuals in the Dolores River, Colorado, study area in an attempt to create synthetic hybrids, and also undertook a comprehensive isozyme analysis of the blue spruce and Engelmann spruce populations in low to high elevations of the drainage. In this work he discovered an isozyme polymorphism that consistently distinguished the two species in this drainage from one another, (PGI-2). This technique was further used to identify hybrids, which possess the characteristic banding patterns of both species.

Although controlled reciprocal crosses between blue spruce and Engelmann spruce had been attempted previously (Fechner and Clark, 1969, Kossuth and Fechner, 1973), none of the resulting hybrids were described morphologically. This condition may have led to circular reasoning in the assignment of hybrid status to individuals based on

morphology alone, a mistake described by Neff and Smith (1979) and Adams (1982).

To overcome this possible problem and to describe the morphology of these hybrids, the present study was undertaken. Specifically, this work has proceeded in two discrete phases. The first phase involved identifying and distinguishing Engelmann spruce x blue spruce hybrid individuals, blue spruce, and Engelmann spruce from Ernst's crosses by examining the PGI-2 phenotype of each individual. After representative groups of each species were assembled, these individuals were then measured for traits which Schaefer found to be diagnostic of the pure species. In addition, growth traits such as height, diameter, terminal bud length and width, and foliage color were measured to compare the appearance and performance of all three groups.

CHAPTER II

DISTINGUISHING BLUE SPRUCE, ENGELMANN SPRUCE AND ENGELMANN SPRUCE X BLUE SPRUCE HYBRIDS BASED UPON PGI-2 PHENOTYPE

ABSTRACT

To assemble groups of known taxonomy for a morphological comparison, the species identity of individual trees was confirmed through electrophoresic examination of the phosphoglucoisomerase (PGI) locus in blue spruce, Engelmann spruce and artificial Engelmann spruce x blue spruce hybrid apical meristematic bud tissue. Polymorphisms of the slower migrating isoenzyme, PGI-2, consistently distinguished the species and hybrids. All existing artificial hybrids, 25 blue spruce, and 25 Engelmann spruce were verified by this method.

INTRODUCTION

Isoenzyme electrophoresis is a valuable tool for biochemical marking or fingerprinting species, varieties, and even individual trees. Allozymes are alternative enzyme forms that are coded for by different alleles at the same locus. Thus, enzyme electrophoresis may distinguish between forms of an enzyme which differ by an amino acid substitution arising from point mutations within DNA or

arising from post translational enzyme modification. Such enzyme polymorphisms may then be used to ascertain the degree of genetic variability between and within populations as well as determine patterns of mating and inheritance (Scandalios, 1969, Andrews, 1983, Smith, 1986).

Concerning the present question of putative natural hybridization between blue spruce and Engelmann spruce, isoenzyme electrophoresis has been used previously in two studies. Mitton and Andalora (1981) used polymorphisms of phosphoglucoisomerase (PGI) to distinguish blue spruce from Engelmann spruce, and found no evidence for hybridization. Ernst (1985) examined 11 enzyme systems in blue and Engelmann spruce of the Scotch Creek drainage in southern Colorado, made controlled crosses of both species in a partial diallel mating scheme, and determined the inheritance of 13 separate loci in these populations. He found that PGI was expressed differently in blue spruce and Engelmann spruce and that synthetic Engelmann spruce x blue spruce hybrids possessed unique PGI isoenzyme phenotypes.

The progeny from Ernst's controlled crosses are the subject of this study, and must therefore be examined individually by electrophoresis to determine species identity.

MATERIALS AND METHODS

Three subject groups comprised of both pure species and their hybrids were assembled as follows: 25 open-pollinated blue spruce progeny, 25 controlled-cross Engelmann x blue spruce hybrid progeny, and 25 controlled-cross Engelmann spruce progeny from the same mother tree as viable hybrids. All individuals are progeny from Ernst's(1985) partial diallel mating and are samples from within the larger group of all surviving offspring grown at the Tree Research Center, Michigan State University.

Tissue samples from each individual were collected during the fall and winter of 1987. Samples consisting of two axillary buds per individual were excised, bagged, placed in styrene vials, and stored in a freezer at -10 degrees celsius until used in electrophoresis.

To extract PGI from bud tissue the bud scales and needle primordia were first removed with a scalpel, exposing the bright green apical dome. This structure, composed of dormant meristematic tissue, was excised and placed in a grinding cuvette for extraction. Each sample of bud meristem tissue was macerated in one ml of extraction buffer and run on two starch gels simultaneously. Each gel also contained samples of two genotypes (one blue spruce and one engelmann spruce) which served as controls. Electrophoresis was carried out on 12.5% starch gels at 330 volts for approximately three hours. The buffers used in preparing

the starch gels were lithium-borate and trisma-citrate, titrated to pH 8.3 after Conkle, et al.(1982). Gels were then stained to reveal PGI isoenzymes, after Conkle. et al.(1982) and Ernst(1985). Precise recipes of the extraction solution, gel and electrode buffers, and staining solution are included in appendix 1. The stained gels were scored, fixed in a 5:5:1 methanol-water-glacial acetic acid solution, and photographed.

This procedure was performed on all hybrid progeny and 25 blue spruce and Engelmann spruce progeny to fulfill the requirements of the experimental design used for a later morphological trait analysis.

RESULTS AND DISCUSSION

In all cases PGI-2 clearly distinguished blue from Engelmann spruce, and both pure species from their F1 hybrids. Figure 1 is a diagrammatic representation of the distinguishing allozyme patterns, and is presented to clarify gel interpretations. Figures 2 and 3 illustrate the actual patterns as seen in blue and Engelmann spruce. Blue spruce phenotype is characterized by banding in the more cathodal (in these figures, lower) regions with a corresponding lack of bands in the anodal (upper) regions of PGI-2. Ernst(1985) considered the bands of blue spruce as genotype 3/3, 3/4, and 4/4, and this pattern was observed in

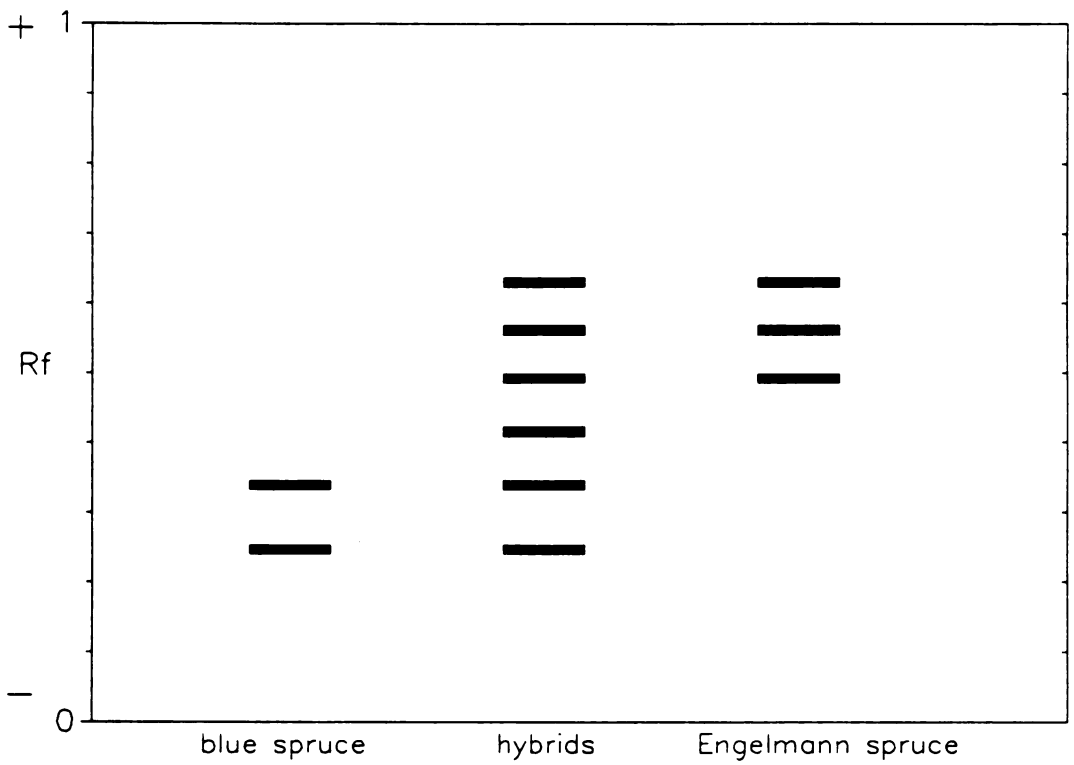

Figure 1. Diagrammatic representation of electrophoretic zymograms of the PGI-2 locus for blue spruce, Engelmann spruce, and their artificial Engelmann spruce x blue spruce hybrids.

Figure 2. Electrophoretic zymogram of individual trees sampled February 23, 1988 (ES - Engelmann spruce signature bands, BS - blue spruce signature bands).

Lane	Accession #	Species classification
1	BS control	BS
2	ES control	ES
3	6731-0794	BS
4	6717-0406	ES
5	6731-0766	BS
6	6717-0385	ES
7	6731-0742	BS
8	6731-0770	BS
9	6731-0758	BS
10	6731-0762	BS

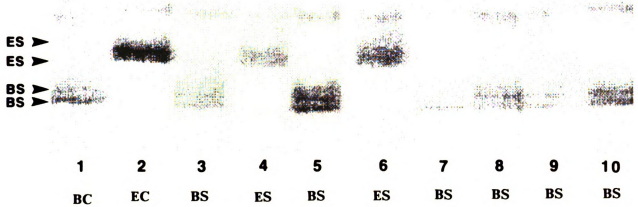

Figure 2.

Figure 3. Electrophoretic zymogram of individual trees sampled February 24, 1988 (ES - Engelmann spruce signature bands, BS - blue spruce signature bands).

Lane	Accession #	Species classification
1	BS control	BS
2	ES control	ES
3	6731-0798	BS
4	6717-0421	ES
5	6717-0404	ES
6	6717-0386	ES
7	6717-0418	ES
8	6717-0370	ES

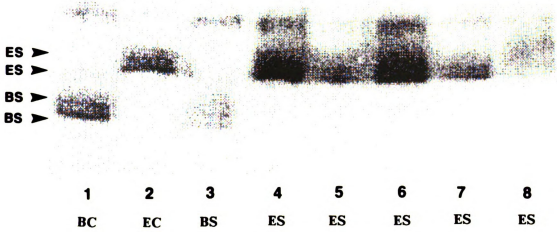

Figure 3.

both the blue spruce control specimens and all blue spruce observed in the current study. Correspondingly, Engelmann spruce was characterized by Ernst(ibid.) as possessing bands in the anodal regions of PGI-2, and these bands were assigned genotypes 1/1, 1/2, and 2/2. All Engelmann spruce used in this study, including the Engelmann spruce control specimens, had this banding pattern. After the blue and Engelmann spruce were confirmed to be true species based upon their PGI-2 phenotype, the synthetic hybrids were easily recognizable. These *Picea engelmannii* x *P. pungens* F1 individuals possess bands in both cathodal and anodal sections of gel, and were previously described by Ernst(ibid.) as genotypes 1/3 and 1/4. Figures 4 and 5 depict this banding pattern, although the bands are somewhat obscured due to fading. The criteria used in assigning hybrid status to an individual was that both anodal and cathodal regions of the gel lane contained bands characteristic of both pure species, not that specific bands could be measured or observed.

Some individuals in this study were reported by Ernst(1988) to be the product of contaminated pollen atches, yet they still possessed accession numbers identifying them as hybrid progeny. For that reason, all individuals with accession numbers representing Engelmann x blue spruce hybrids were subjected to the PGI-2 zymotyping process. Individuals found to be products of pollen contamination (Engelmann spruce) were discarded from the

Figure 4. Electrophoretic zymogram of individual trees sampled February 9, 1988 (ES - Engelmann spruce signature bands, BS - blue spruce signature bands).

Lane	Accession #	Species classification
1	BS control	BS
2	ES control	ES
3	6761-0133	HY
4	6761-0117	HY
5	6761-0082	HY
6	6761-0135	HY
7	6761-0082	HY
8	6761-0130	HY
9	6761-0118	HY
10	6761-0127	ES

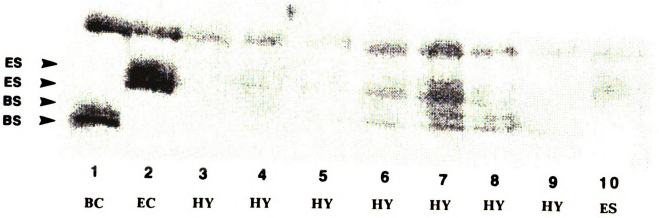

Figure 4.

Figure 5. Electrophoretic zymogram of individual trees sampled July 19, 1988 (ES - Engelmann spruce signature bands, BS - blue spruce signature bands).

Lane	Accession #	Species classification
1	BS control	BS
2	ES control	ES
3	6761-0081	HY
4	6761-0119	HY
5	6761-0081	HY

Figure 5.

study and destroyed. Many individuals were found to be Engelmann progeny, but all of these were noted by Ernst as such. Only one individual not noted in Ernst's communication was found to be a true hybrid, and this tree was retained for the following morphological study along with other verified hybrids.

Interestingly, the polymorphisms at the PGI-2 locus were also used in a study by Mitton and Andalora(1981), in which blue and Engelmann spruce could be consistently separated in the same fashion. That is, the slowly migrating alleles were apparent in blue spruce exclusively, while faster migrating alleles were representative of Engelmann spruce. As expected, no naturally occurring hybrids (genotypes 1/3 or 1/4) were identified by them through this technique. (Fechner and Clark, 1969, Kossuth and Fechner, 1973, Schaefer and Hanover, 1985,1987,1990).

The identity of the hybrids was further supported by the work of Stine(1988), in which the inheritance of cpDNA was shown to be paternal in species of *Picea*. The study hybrids possessed blue spruce cpDNA, and are therefore not artefacts (ibid.).

CONCLUSIONS

The results presented here agree and are consistent with the results obtained by Ernst(1985). The PGI-2 polymorphisms that he described as being characteristic of

pure species and hybrids are apparently stable throughout the juvenile growth stage of development, and therefore are a valuable tool in recognizing F1 hybrids from the Dolores River / Scotch Creek, Colorado, populations, should they exist. The banding patterns observed were unambiguous and consistent. The relative simplicity of the technique involved in distinguishing species will facilitate more comprehensive, possibly range wide, studies of the interactions between blue and Engelmann spruce in the future.

CHAPTER III

MORPHOLOGICAL AND GROWTH TRAIT ANALYSIS OF BLUE SPRUCE, ENGELMANN SPRUCE AND ARTIFICIAL ENGELMANN X BLUE SPRUCE HYBRIDS

ABSTRACT

Four foliage traits (needle width, needle thickness, maximum number of stomatal lines, and needle sharpness) which distinguish blue spruce from Engelmann spruce were measured in 25 artificial Engelmann spruce x blue spruce hybrids, 25 open pollinated blue spruce progeny, and 25 Engelmann spruce progeny. These measurements were used in analyses of variance, linear regression, and canonical variates discriminant function analysis. All group means were significantly different from each other for each trait, and the hybrid group was always quantitatively intermediate, yet there was a great degree of overlap between taxa. The three quantitatively measured traits (all except sharpness) were strongly positively correlated with one another. Discriminant function analysis separated blue and Engelmann spruce, but failed to distinguish hybrids from pure species with certainty.

Foliage color, height, diameter, and terminal bud length and width were also measured in the three study groups. Analyses of variance revealed no clear pattern of inheritance of these traits in the hybrids, although significant differences between group means were found in terminal bud length and tree height.

INTRODUCTION

Blue spruce and Engelmann spruce are morphologically quite similar which has led many authors to search for distinguishing traits that could be easily observed in the field to aid in distinguishing the species. These traits range from foliage, twig, and bark characteristics to crown appearance and cone characteristics such as cone length and cone scale shape and size. Cone characteristics have been described by Daubenmire (1972), Taylor et.al. (1975), and Schaefer and Hanover (1985, 1990) as the most useful of traits to distinguish the two species from one another. Next in utility come the needle traits, including the number of stomatal rows, resin sac abundance, size and shape of foliage, and sharpness (Marco, 1931, Reed and Freytag, 1949, Daubenmire, 1972, Mitton and Andalora, 1981, Schaefer and Hanover, 1985, 1990). Finally, twig pubescence and color, branching habit, bark texture, and crown appearance have all been shown to have some value in diagnosing specific identity (Taylor, et.al., 1975, Jones and Bernard, 1977, Shaefer and Hanover, 1985, 1990).

This extensive list of diagnostic traits has been used in attempts to identify naturally occurring hybrids which may possess intermediate expression of these characteristics relative to the two species. In Anderson's (1949) treatise on hybridization, such combinations of phenotypes would be expected to appear in the F1 generation of hybrids, and

these hybrids would be expected to be found in disturbed, intermediate environments for the species. Since the Engelmann spruce x blue spruce hybrids are extremely rare in nature (none have been identified), trees that possess intermediate phenotypes based on a combination of traits have been the main target for research regarding natural hybridization.

The methods most commonly employed to identify hybrids involve the construction of a hybrid index, derived from values of measured traits in the pure species, by which intermediate individuals fall into a 'hybrid' category. Although such indices have the potential to identify putative hybrids, the misuse of the technique may lead to erroneous conclusions regarding the frequency and characteristics of natural hybridization. By measuring diagnostic traits in known hybrids along with progeny of both blue and Engelmann spruce from well defined populations, I hope to avoid the pitfalls associated with hybrid morphological indices, while portraying an accurate and informative representation of true Engelmann spruce x blue spruce hybrids.

MATERIALS AND METHODS

In the fall and winter of 1987 secondary branches containing needle growth from the previous summer were

collected from 25 artificial hybrids, 25 blue spruce and 25 Engelmann spruce. The buds at the tips of these branches were used in the electrophoretic analysis described previously, so all individuals were positively identified as being a pure species or hybrid. After the buds were excised for electrophoresis three needles of 1987 growth were randomly selected, excised from the stem, and the following traits measured: Needle width, needle thickness, and maximum number of stomatal lines summed over all four sides of the needle. A digital caliper was used to measure needle width and thickness, which were recorded to the nearest 0.05 mm. To accurately count stomatal lines, all that was required was a hand held 10x magnifying glass.

In the spring of 1988, before the seedlings began new growth, they were lifted from the nursery beds and transplanted to three gallon containers. At this time measurements of terminal bud diameter and terminal bud length were measured on all trees in the collection and recorded to the nearest .1 mm. After bud burst, growth, and subsequent bud set, foliage sharpness and color were subjectively measured in the 25 representatives of each species group on a qualitative scale of 1 to 3, 1 representing soft foliage or green color and 3 representing sharp foliage or blue color. After the 1988 bud set, seedling height (in centimeters) and stem diameter (in millimeters) was measured for all trees in the collection.

An analysis of variance was performed on all data of

the three quantitative needle traits based on the completely randomized design with sub-sampling model (species, t = 3, individuals per species, r = 25, needle sample, s = 3). For foliage color and foliage sharpness traits, the analysis of variance design was a completely randomized design with no sub-sampling (species, t = 3, individuals per species, r = 25). For the growth traits, the unbalanced data set was analyzed through the general linear models routine of the Statistical Packages for Social Sciences, version 10 (SPSSX). Canonical discriminant function analysis was also performed through SPSSX. Graphics were created with Plotit, and produced at the computer graphics laboratory, Michigan State University.

RESULTS AND DISCUSSION

Significant differences exist between blue, Engelmann, and Engelmann x blue spruce in characters that successfully distinguish the pure species apart, namely needle sharpness, maximum number of stomatal lines, needle width and needle thickness. But even though these mean differences are significant, there is still a large degree of overlap in these traits.

Analysis of variance for these four traits are given in table one and table 2 presents the multiple range test results revealing significant differences. In the analysis

Table 1. Degrees of freedom and mean squares showing the significance of the variation between blue, Engelmann, and Engelmann x blue hybrid spruce for 4 diagnostic characteristics.

	DF		Mean Square	
Characteristic	Between groups	Within groups	Between groups	Within groups
needle sharpness	2	72	14.5***	.25
# stomatal lines	2	222	325.8***	3.23
needle width (.01mm)	2	222	16640.3***	140.19
needle thickness (.01mm)	2	222	2849.8***	98.43

*** - significant at .001 level of probability

Table 2. Least significant difference multiple range test of means for blue, Engelmann and hybrid Engelmann x blue spruce diagnostic characteristics, at .001 level of probability.*

	Species Means		
Characteristic	Engelmann	Hybrid	Blue
needle sharpness**	1.28[a]	1.96[b]	2.80[c]
# stomatal lines	10.9[a]	12.7[b]	15.1[c]
needle width (.01mm)	83.3[a]	96.5[b]	113.0[c]
needle thickness (.01mm)	66.9[a]	71.9[a]	79.1[b]

* Means followed by the same letter are not significantly different at the .001 level of probability.

** 1 = dull, 2 = intermediate, 3 = sharp

of variance, each measurement of a character in an individual was pooled with the measurements of other individuals in a species group. This is due to the fact that after analysis of the subsampling design results, it became clear that sampling error was very small and statistically insignificant, as was sample by species interaction. For the purposes of this study it is reasonable to combine sample measurements within the species group, since no special weighting is given to any one individual and the number of individuals and observations per individual are the same in all groups. The design of the analysis is therefor a completely randomized design, with each tree yielding three observations. In either case, the differences between the means of species was highly significant. The assumption of equality of variance between the groups was tested by Bartlett's F test, which for all traits revealed no significant differences in variances.

For the multiple range tests, the least significant difference test was chosen because in SPSSX it could be used at the .001 level of probability. The results in table 2 indicate that the pattern of inheritance for characters of diagnostic value is one of intermediacy and not paternal or maternal. Only needle thickness failed to discriminate between all three groups of individuals at this high level of probability. This confirms the assumptions made by Schaefer and Hanover(1990) that putative hybrids would be expected to display intermediacy in these traits, and agrees

with the vast majority of research regarding hybrid needle characteristics in the family Pinaceae (Mergen, 1958, Keng and Little, 1962, Critchfield, 1964, Silen et.al.,1965, Wright et.al., 1969).

The results of diagnostic character measurements are graphically represented by histograms in figures 6 through 9, showing the range and degree of overlap in individuals as well as location of the hybrid group data in relation to the two species. For the three quantitatively measured traits each group appears normally distributed around the means for each group, with the hybrids occupying an intermediate position in relation to the blue spruce and Engelmann spruce groups. The one qualitatively measured diagnostic trait, needle sharpness (figure 9), distinguished blue spruce from Engelmann spruce quite well, as no Engelmann spruce was rated 'sharp' while no blue spruce was rated 'soft'. Each species did display a small amount of intermediacy in this trait, as did the majority of the hybrids.

The results of the diagnostic trait measurements indicate that there are severe limitations to extrapolating the specific identity of an individual from such measurements. The most important of these limitations arises from the large degree of morphological overlap of traits between the two species and the hybrids. The means of small (n = 25) populations may show significant differences in these diagnostic characteristics and distinguish a hybrid group well, but would these differences

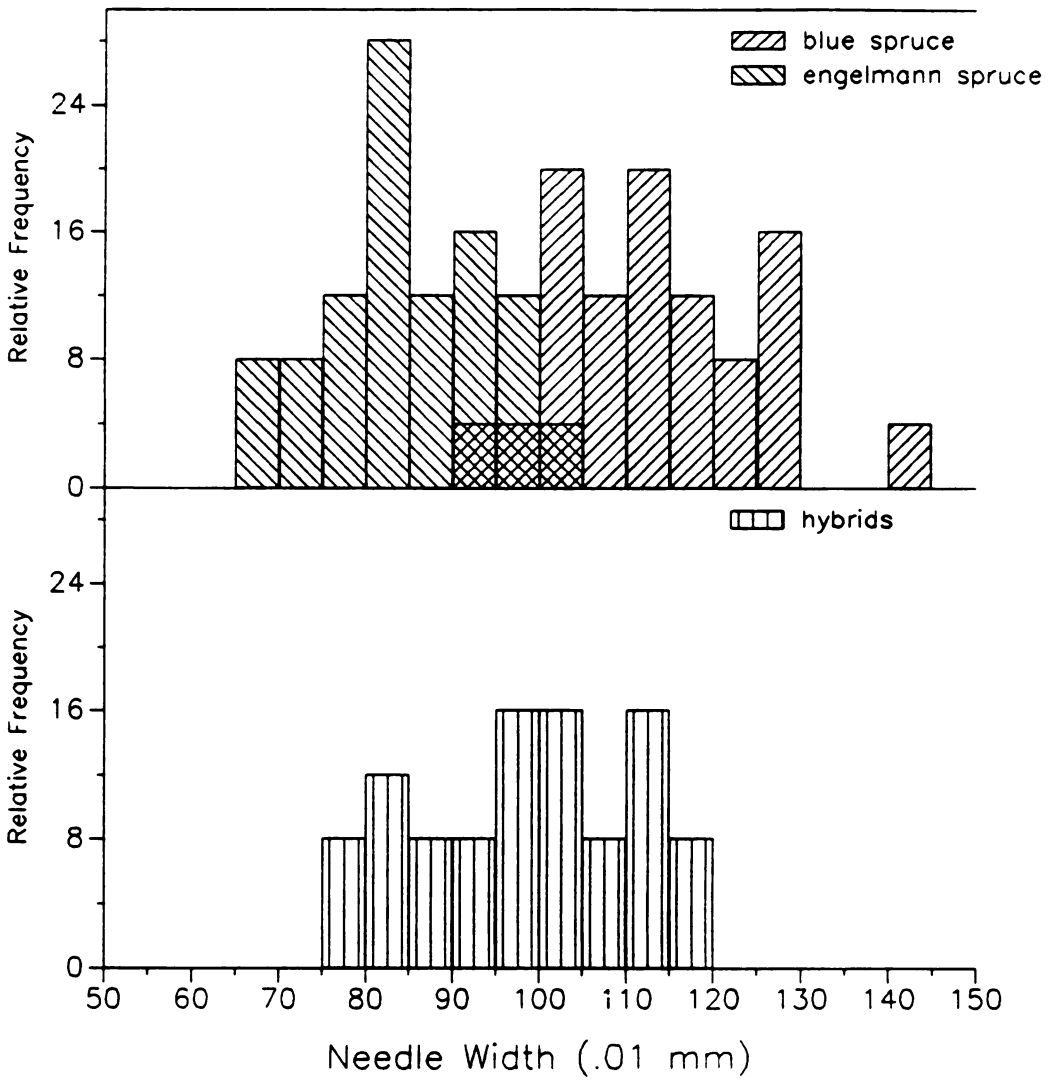

Figure 6. Relative frequency histogram of needle width for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

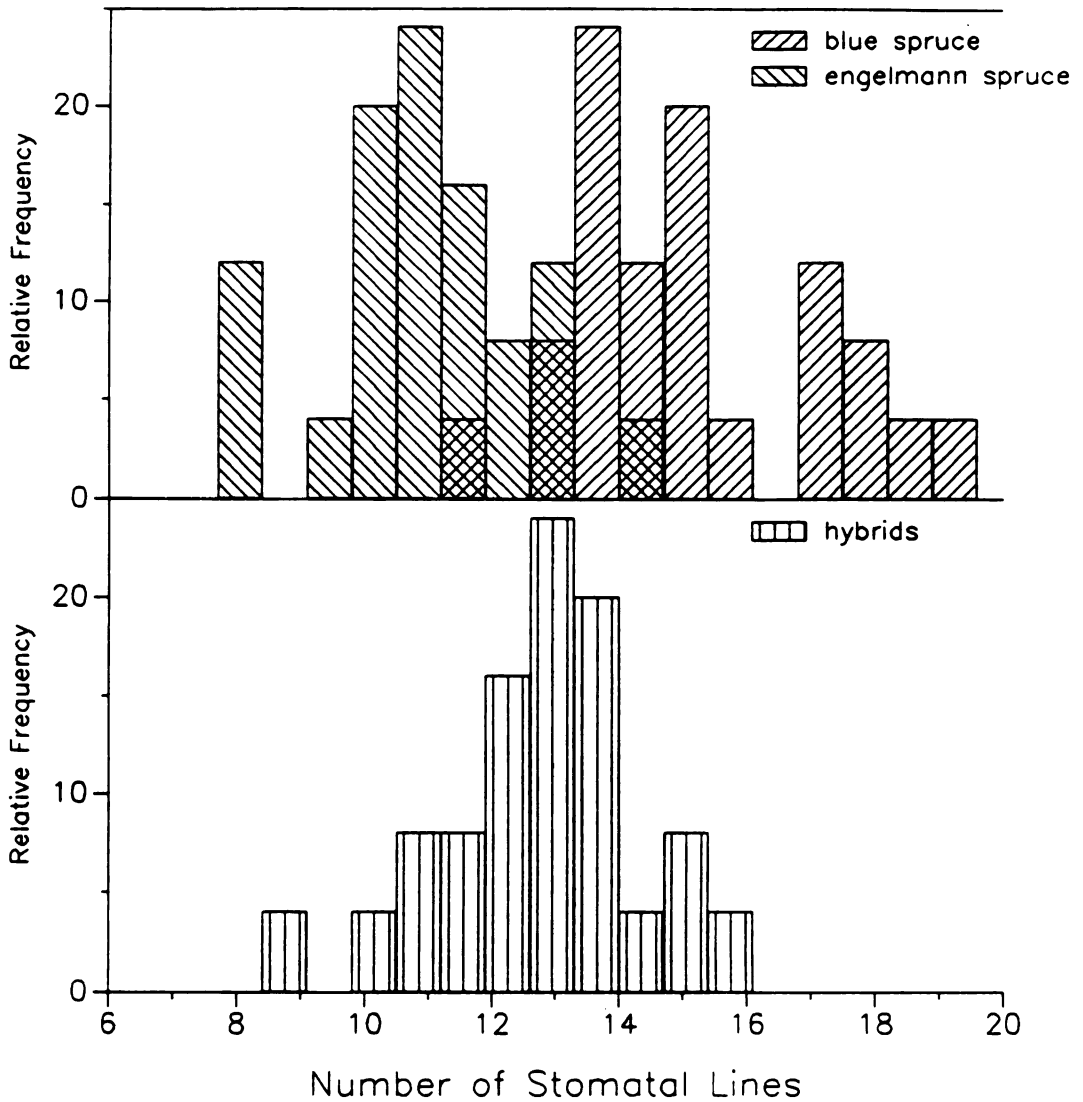

Figure 7. Relative frequency histogram of maximum number of stomatal lines for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

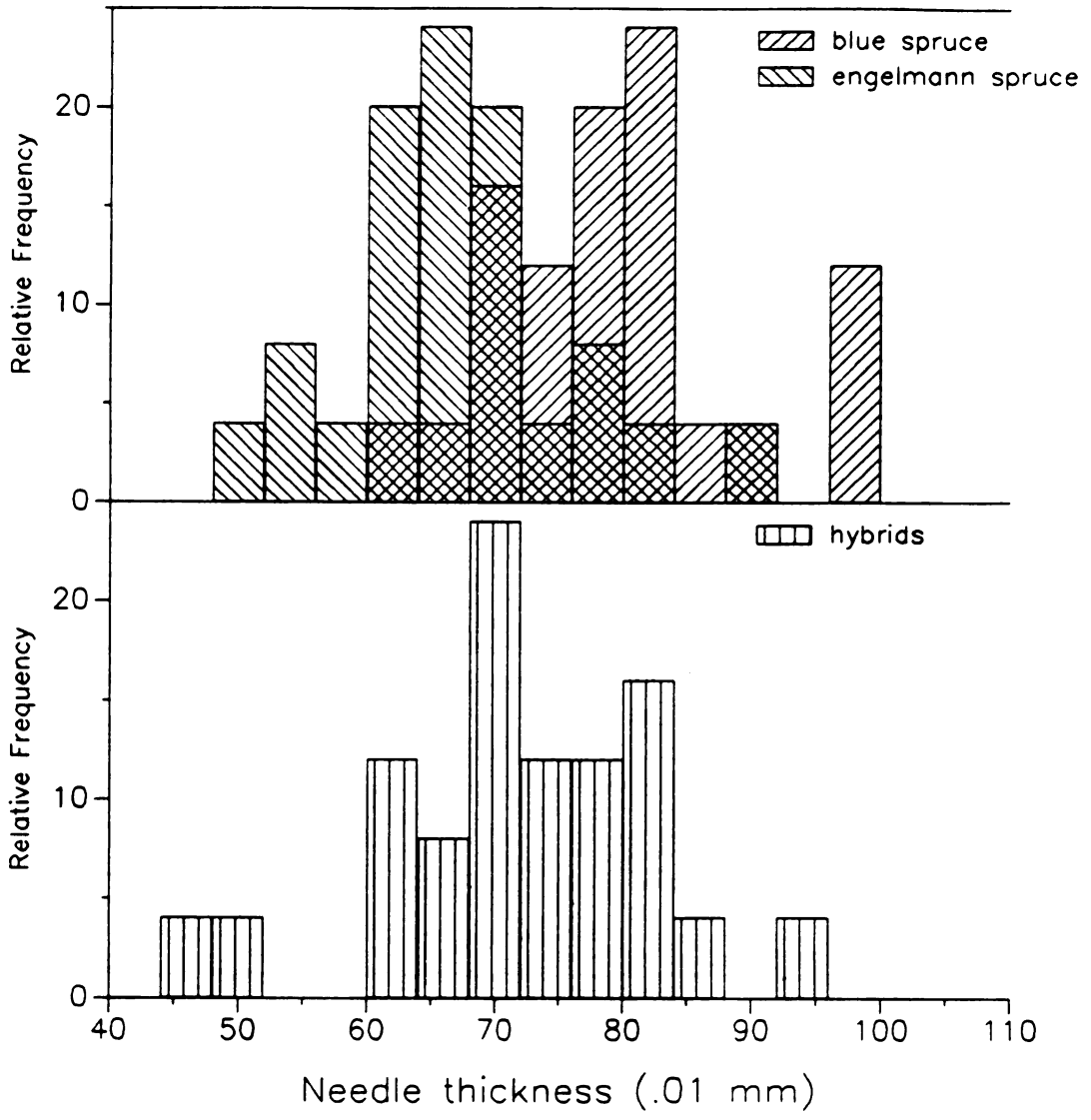

Figure 8. Relative frequency histogram of needle thickness for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

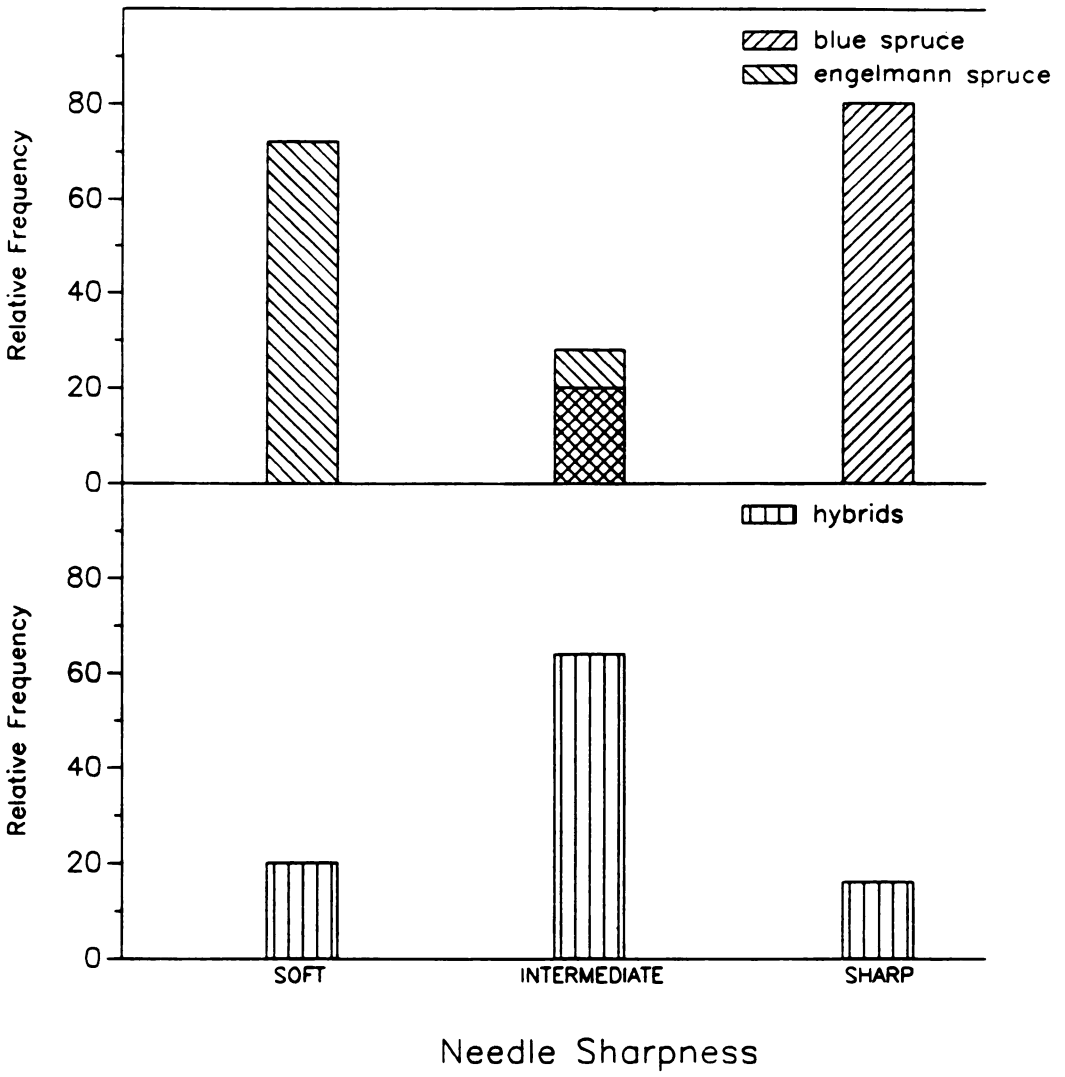

Figure 9. Relative frequency histogram of needle sharpness for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

consistently distinguish an individual of one group from another? They probably would not.

Figures 10 through 12 illustrate the last point. These figures represent the three quantitatively measured traits (number of stomatal lines, needle width, and needle length) for each group. The notchbox for each comparison supplies the important information of range of values, median (the horizontal center line), and 95 percent confidence interval for the median (the angled lines emanating from the median line). These plots clearly show that the degree of overlap of outlying points in each group is enough to theoretically include that point in any one of the groups without a priori group assignment. Even though the confidence intervals between groups in general remain distinct, this overlap can render individual tree measurements almost meaningless for verification of hybridity.

To illustrate the relationship between quantitative traits, pairs of the traits were plotted against each other along with the best fit regression line calculated by Plotit in figures 13 through 15. The correlation coefficient r is also given for these data. All three traits were positively correlated with one another; needle width and needle thickness being the most strongly correlated traits ($r = .8297$) and needle thickness and number of stomatal lines being the most weakly correlated traits ($r = .5941$). That the correlation should be positive in these traits is no surprise, since overall needle size dictates such

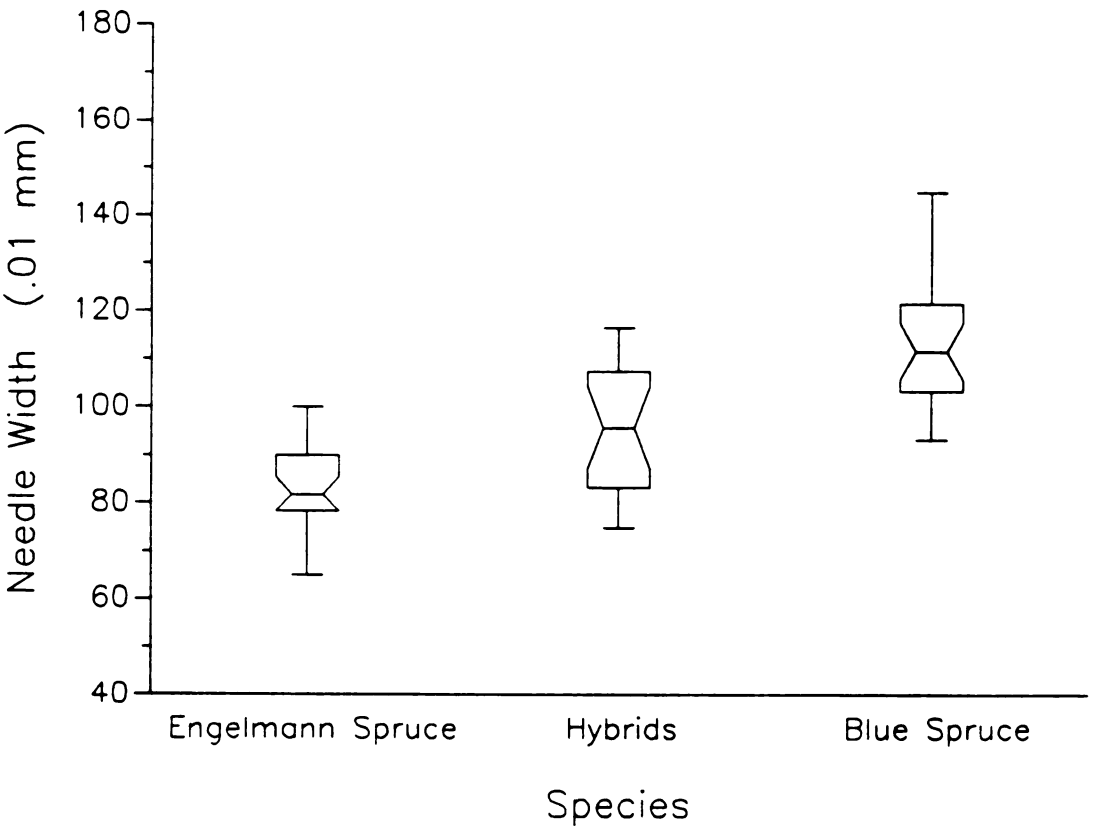

Figure 10. Notchbox graph of needle width for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce. Each symbol depicts the range, median and 95 percent confidence interval of the mean for each taxon.

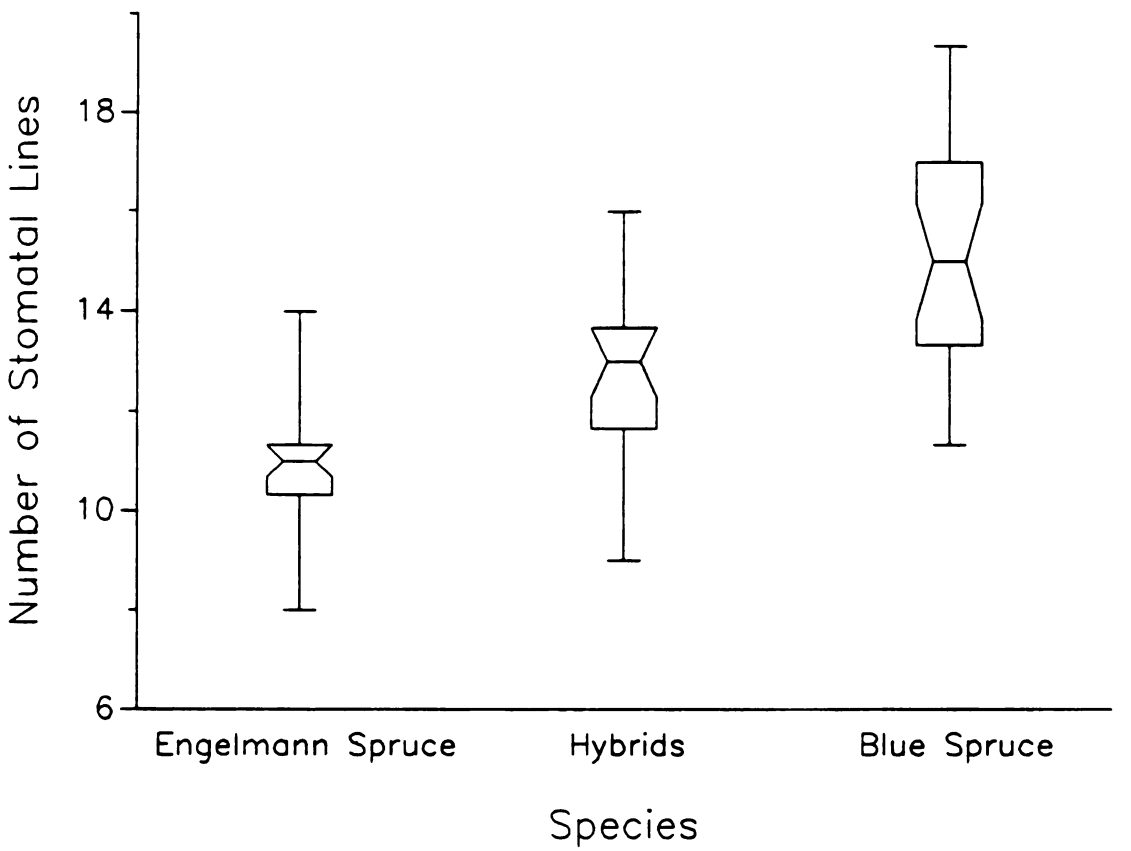

Figure 11. Notchbox graph of maximum number of stomatal lines for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce. Each symbol depicts the range, median and 95 percent confidence interval of the mean for each taxon.

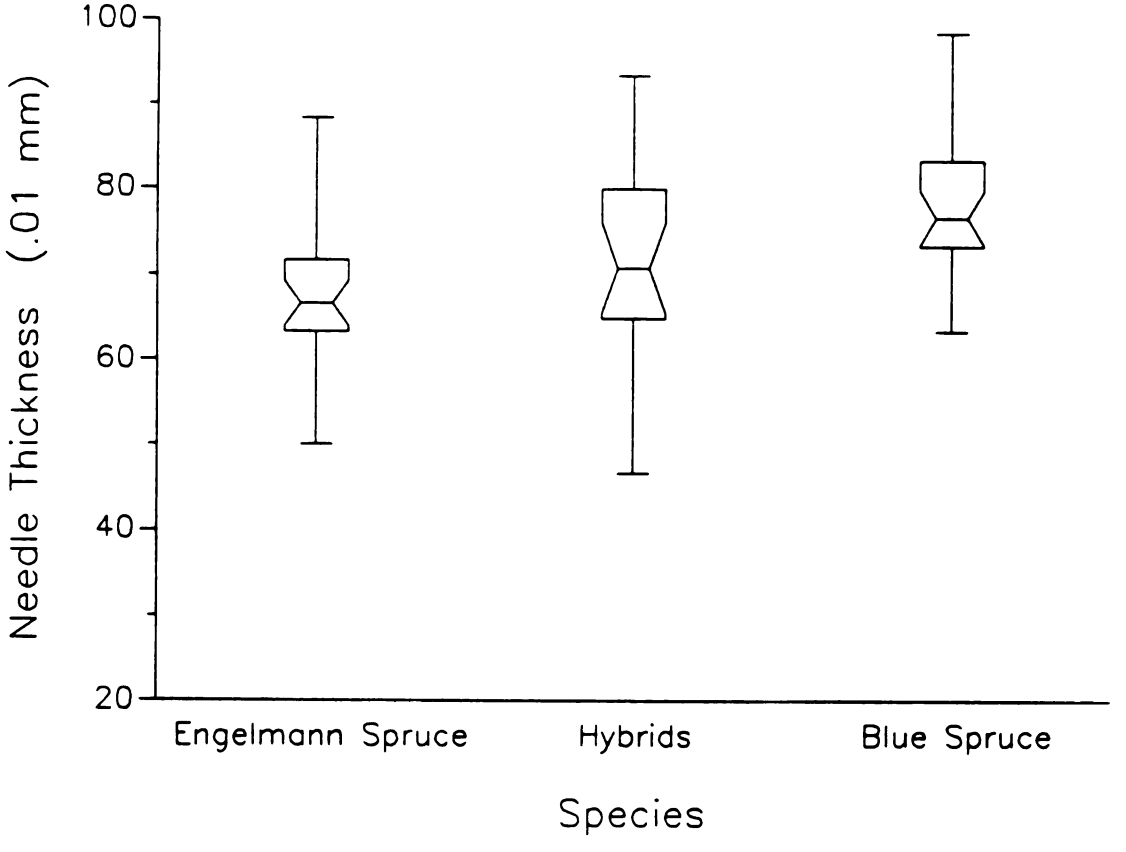

Figure 12. Notchbox graph of needle thickness for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce. Each symbol depicts the range, median and 95 percent confidence interval of the mean for each taxon.

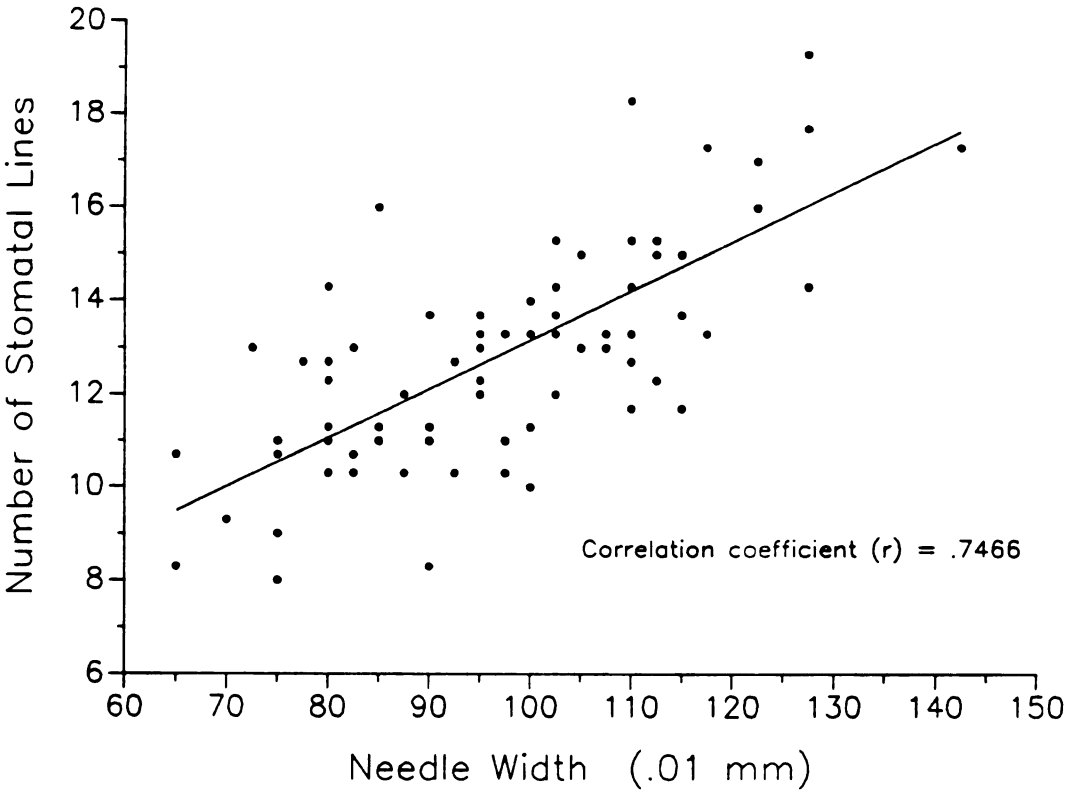

Figure 13. Needle width - stomatal line regression line for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

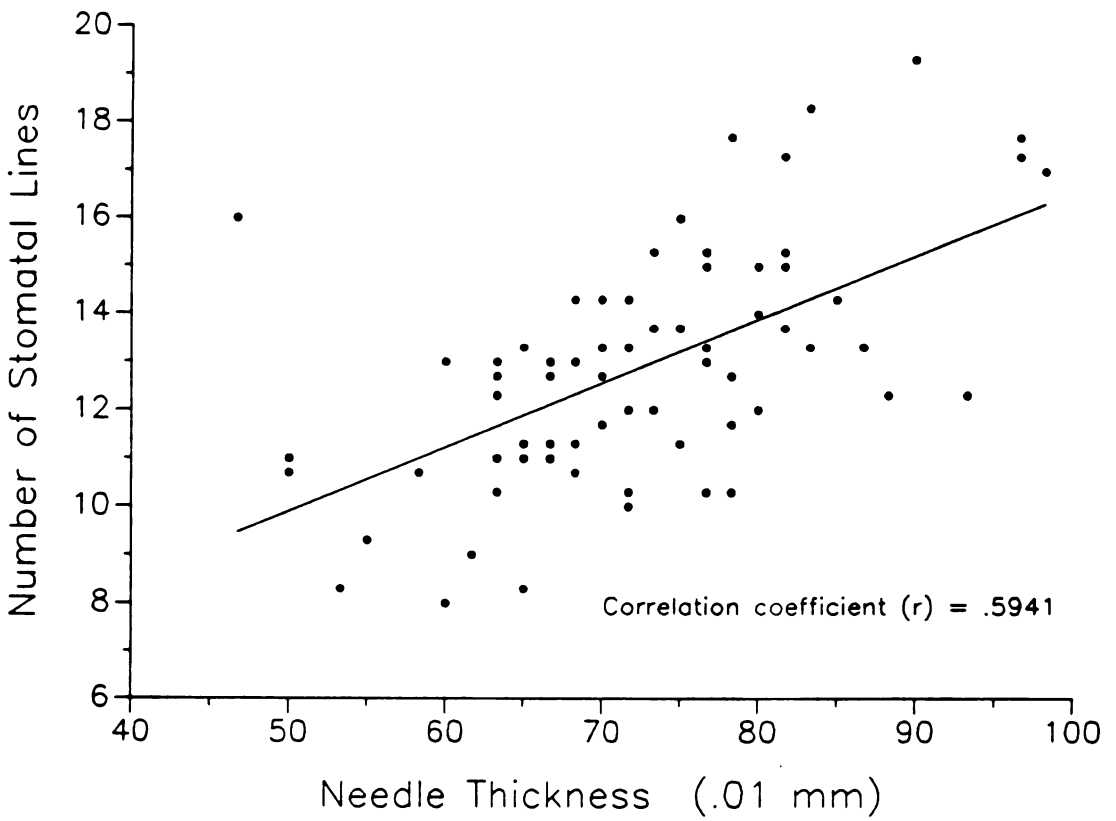

Figure 14. Needle thickness - stomatal line regression line for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

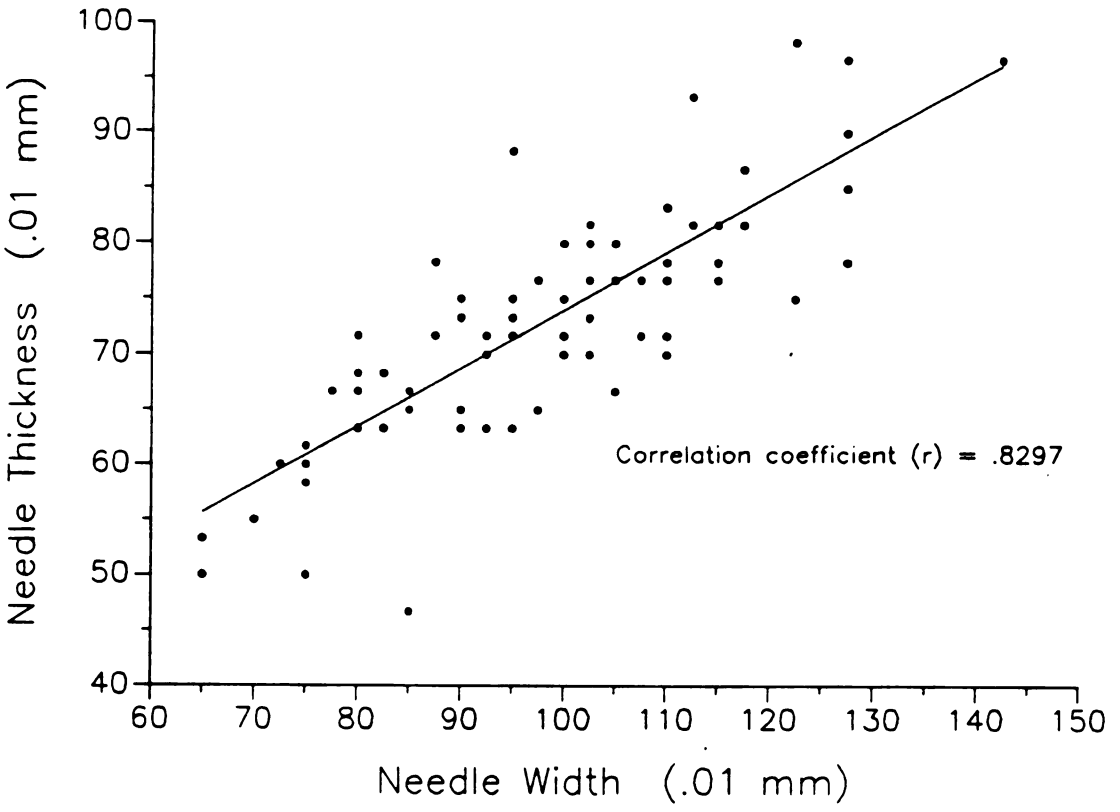

Figure 15. Needle width - needle thickness regression line for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

parameters. The less than perfect association of these characters represents differing needle phenotypes in shape and stomate distribution, arising from species differences as well as possible physiological effects of needle position within the branch of an individual tree, although the effect of sampling could be considered rather small.

In any case it is reasonable to assume that needle characteristics are inherited in groups or blocks. This finding supports the work of Clausen et.al.(1940,1947,1948) in which the phenomenon of coherence of traits is described. Although only needle characteristics were measured in the present study, other characteristics involving reproduction and other adult physiological processes which distinguish blue from Engelmann spruce may be inherited as groups. The understanding of the pattern of inheritance of characteristics should lead to a broader appreciation for the evolutionary dynamics of the blue spruce divergence from the Engelmann spruce progenitor species.

The use of diagnostic traits in combinations to identify hybrids of blue spruce and Engelmann spruce based upon an index has been attempted several times previously (Daubenmire, 1972, Taylor et.al., 1975, Mitton and Andalora, 1981, Schaefer, 1985, Schaefer and Hanover, 1985,1987,1990), but never before has it been possible for a hybrid group to be assigned *a priori* to such a study. With *a priori* group assignment, canonical variate discriminant analysis becomes a suitable tool to describe and rank the importance of

discriminating variables (Dancik and Barnes, 1975). Results of canonical variate analysis of the data for the four diagnostic characters are given in figure 16. Within function correlations and the discriminant function coefficients of the analysis are shown in table 3 and table 4 illustrates predicted group membership based upon the discriminant functions. Needle sharpness, needle width, and number of stomatal lines contributed greatly to the first discriminant function, which accounted for almost all the variation in the analysis, while needle thickness contributed much less to the discrimination of groups. Group membership predictions based upon the analysis show that while blue and Engelmann spruce are reliably distinguished from one another by these traits, there remains a better than 1 in 4 four chance that an F1 hybrid will be classified as one of the pure species if only these traits are used.

The results of color and growth trait measurements are summarized in tables 5 and 6. These measurements were made to compare hybrid progeny with blue and Engelmann spruce progeny in early growth stages in a uniform environment, and to possibly uncover heterosis or hybrid dysgenesis in juvenile trees. The number of observations for these traits differs because they were not all measured at the same time, and mortality of individuals occurred between bud measurements and height and diameter measurements. In the growth comparisons all living individuals were measured (not

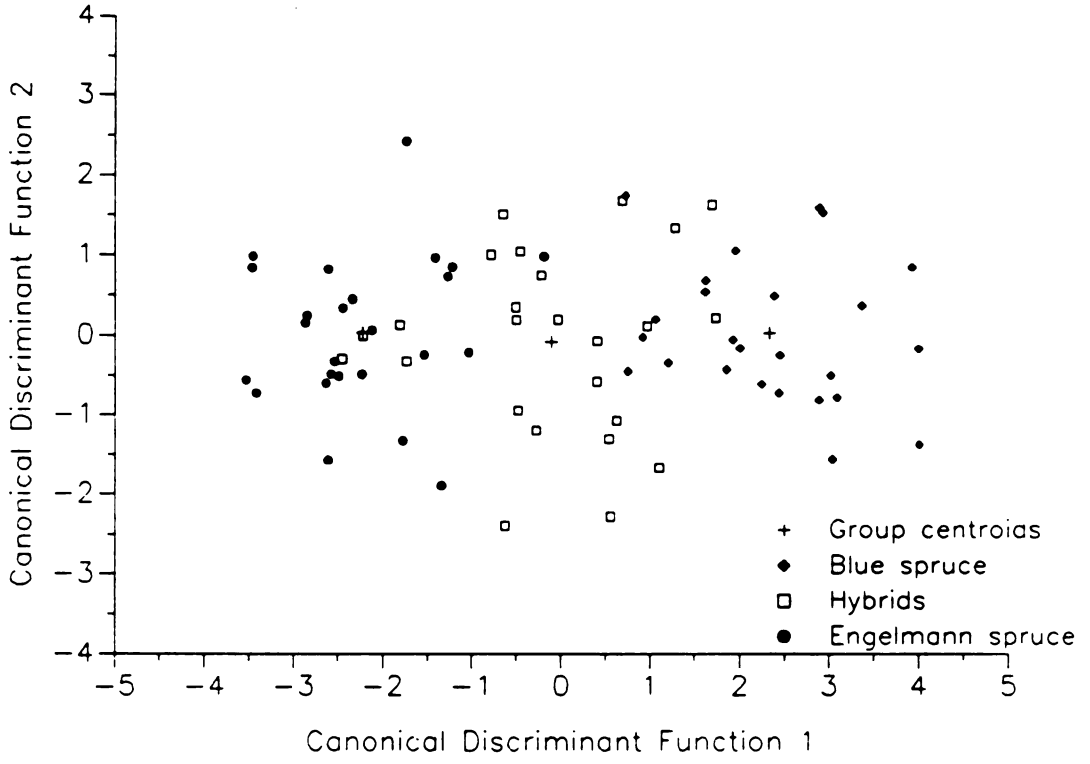

Figure 16. Plot of canonical discriminant function 1 (horizontal) vs. canonical discriminant function 2 (vertical) for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

Table 3. Factor loadings and discriminant function coefficients from diagnostic traits analyzed in blue, Engelmann, and Engelmann x blue hybrid spruce.

	Principal component axes		Discriminant function coefficients	
Characteristics*	I	II	I	II
needle sharpness**	.667	.448	.7510	.2162
needle width (.01mm)	.560	-.001	.8986	-1.2518
# stomatal lines	.531	.333	.4364	.3124
needle thickness (.01mm)	.285	.579	-.8266	1.3717
Variation explained			99.9%	.1%

*Characters ordered by size of correlation within function.

**1 = dull, 2 = intermediate, 3 = sharp

Table 4. Classification results from canonical discriminant function analysis of diagnostic morphological traits.

	Predicted group membership		
Species*	Engelmann	hybrid	blue
Engelmann spruce	23 (92%)	2 (8%)	0
hybrids	4 (16%)	18 (72%)	3 (12%)
blue spruce	0	4 (16%)	21 (84%)

Percent of 'grouped' cases correctly classified: 82.7%

*Assignment based upon previous isoenzyme work.

Table 5. Degrees of freedom and mean squares showing the significance of variation between blue, Engelmann, and Engelmann x blue hybrid spruce for color and growth traits.

	DF		Mean Square	
Characteristic	Between groups	Within groups	Between groups	Within groups
color	2	72	.160^{ns}	.369
terminal bud length (mm)	2	186	75.8***	1.46
terminal bud width (mm)	2	186	22.7***	2.07
tree height (cm)	2	174	1700.0***	70.2
tree diameter (cm)	2	174	56.7**	9.80

^{ns} - differences between groups not significant
** - significant at .01 level of probability
*** - significant at .001 level of probability

Table 6. Least significant difference multiple range test of means for color and growth traits of blue, Engelmann, and Engelmann x blue spruce (.001 level of significance*)

	Species means		
Characteristic	Engelmann	Hybrid	Blue
foliage color**	2.12^{a}	1.96^{a}	2.04^{a}
terminal bud length (mm)	6.82^{a}	7.22^{a}	8.74^{b}
terminal bud width (mm)	6.35^{a}	6.55^{ab}	7.39^{b}
tree height (cm)	35.1^{a}	41.9^{b}	44.7^{b}
tree diameter (mm)	12.6^{a}	13.3^{a}	11.3^{a}

* Means followed by the same letter are not significantly different at the .001 level of probability.

just 25 representatives from each group), in order to include the widest range of possible measurements from each group.

Foliage color did not differ significantly between the three taxons; all means for this trait were close to the 'intermediate' level of bluishness. In general, the Engelmann spruce progeny were somewhat more blue than the blue spruce progeny, reflecting the nature of the Dolores River, Colorado, populations from which these progeny were derived (Hanover,pers. comm.). However, foliage color is not considered a diagnostic trait (Schaefer and Hanover, 1985). Figure 17 illustrates the results of the qualitative foliage color measurements.

Terminal bud characteristics were significantly different in the analysis of variance. Terminal bud length appears to be under some maternal influence in the hybrid spruce, as both hybrid and Engelmann spruce differed significantly from blue spruce in this regard. That result may also reflect a flaw in the design of this comparison, however, due to the fact that all the Engelmann and hybrid progeny are maternally related whereas blue spruce progeny are open pollinated and thus probably not related genetically to the other two groups. Terminal bud width was also significantly different between blue and Engelmann spruce, but in this case the hybrids were not significantly different from either pure species. Figures 18 and 19 show these bud characteristic measurement results.

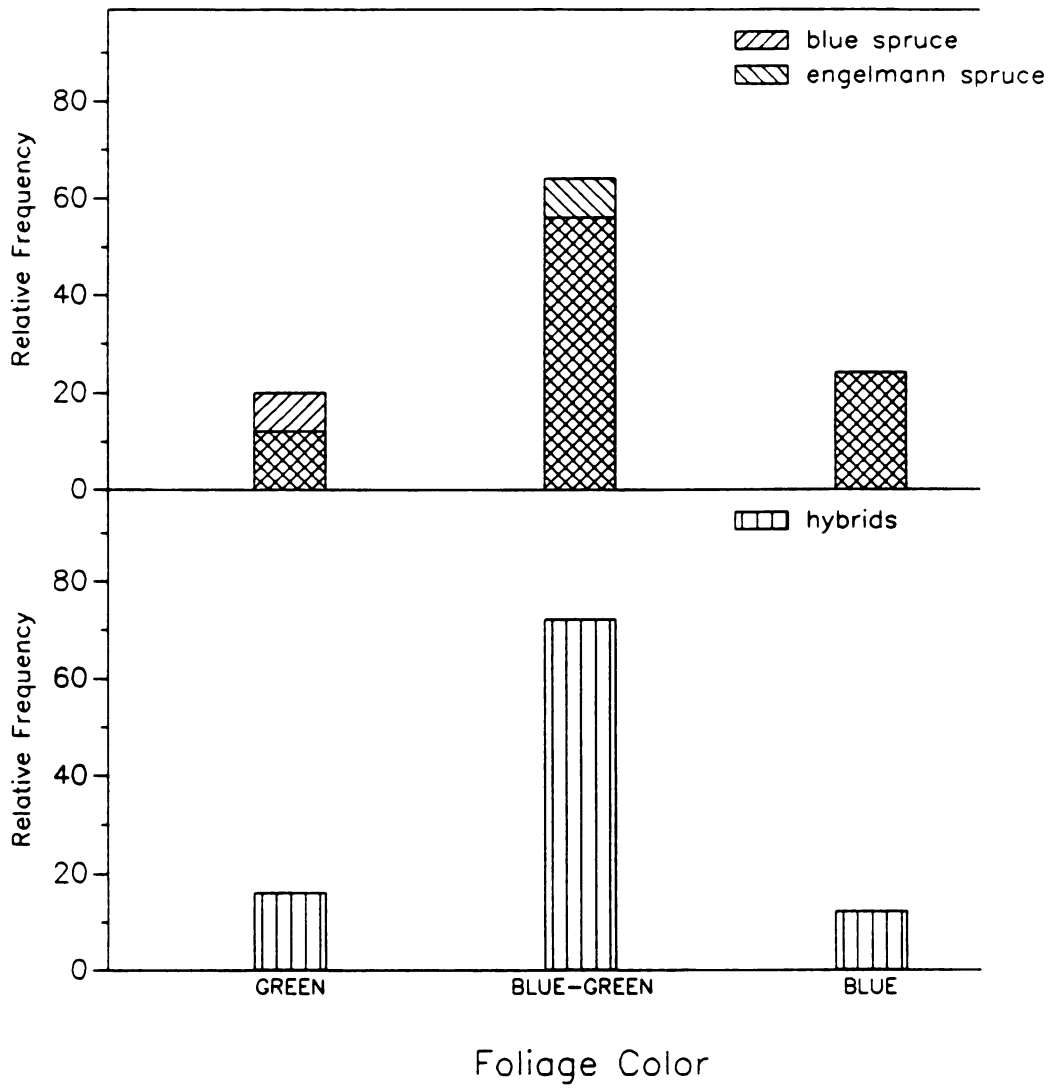

Figure 17. Relative frequency histogram of foliage color for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

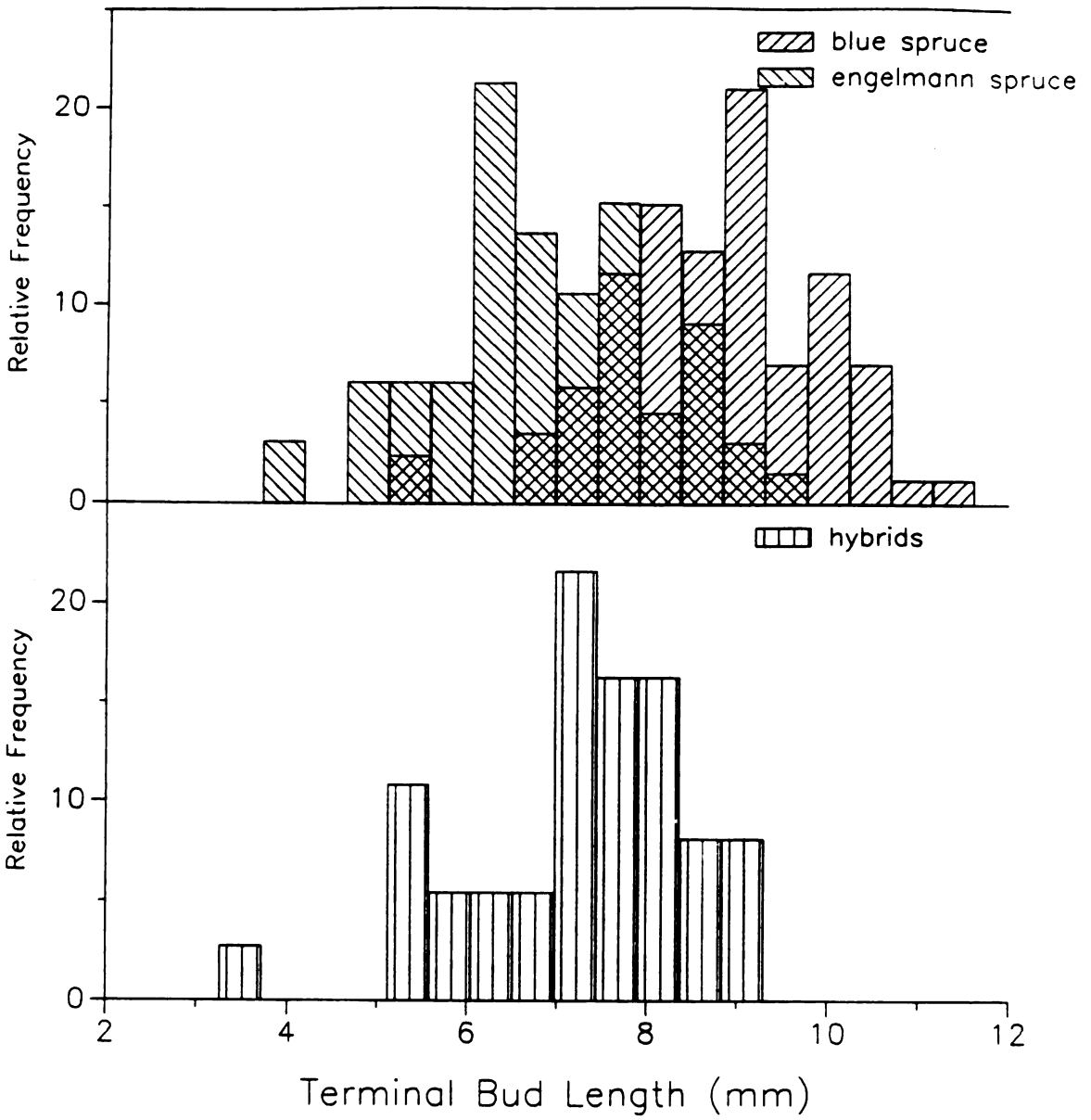

Figure 18. Relative frequency histogram of terminal bud length for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

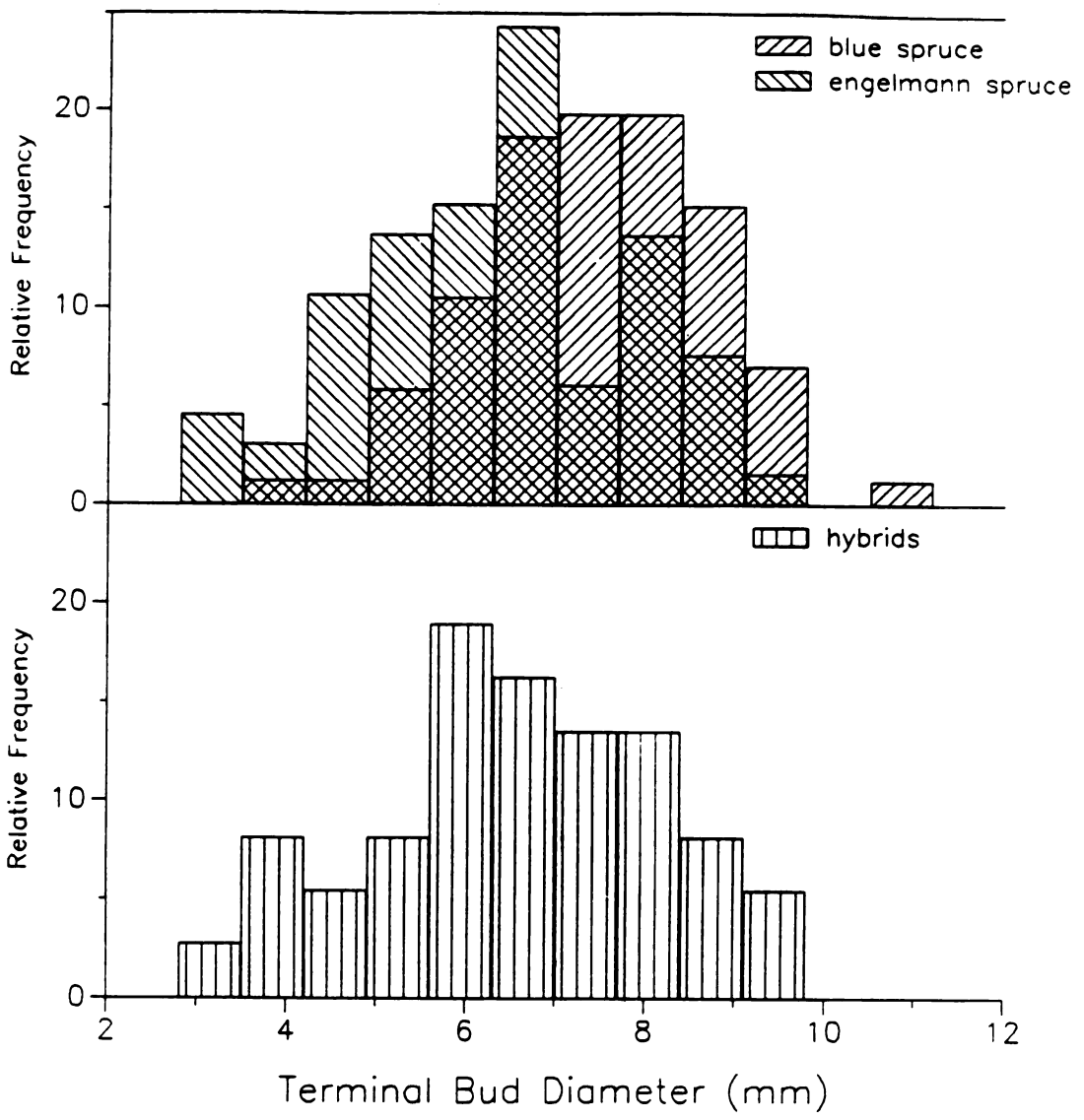

Figure 19. Relative frequency histogram of terminal bud diameter for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

The analysis of tree height and diameter measurements revealed significant differences between group means. In the case of height, hybrids resembled blue spruce while Engelmann spruce differed significantly from both. This result may be due to less winter burn for blue and hybrid spruce in Michigan as compared to Engelmann spruce, although this observation is purely anecdotal and non-quantitative. No group means differed significantly from one another in diameter measurements. Height and diameter measurements are illustrated in figures 20 and 21.

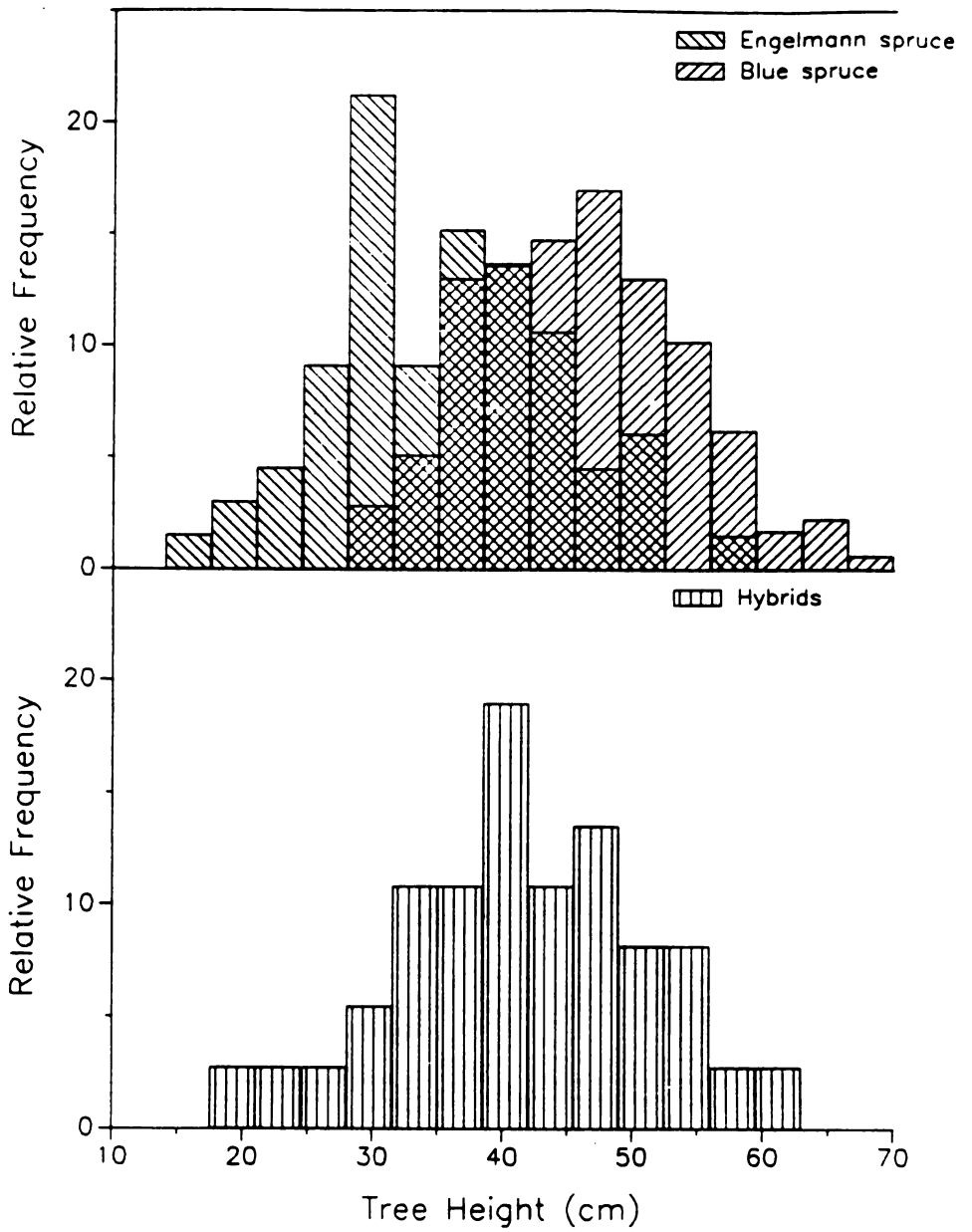

Figure 20. Relative frequency histogram of tree height for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

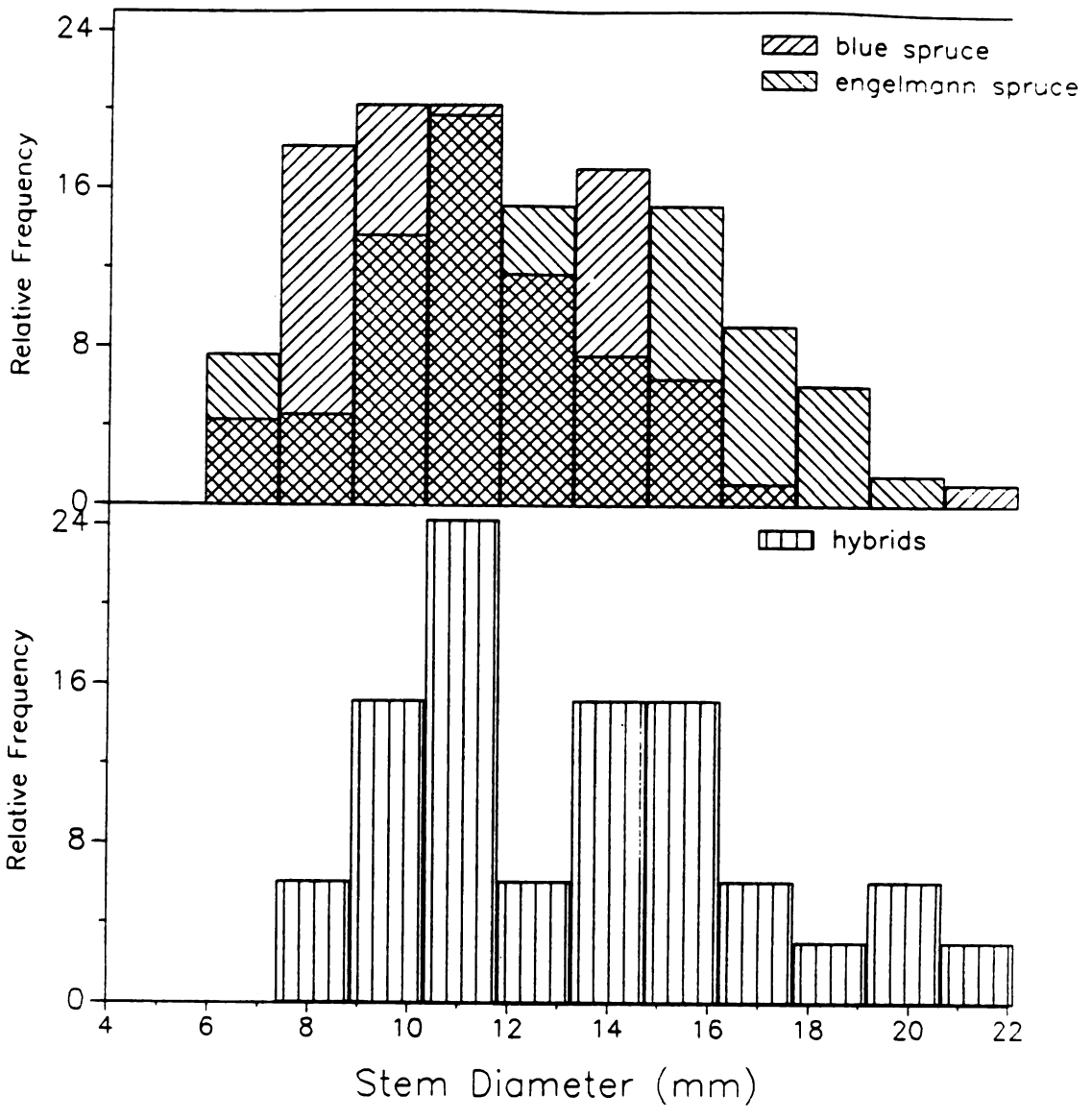

Figure 21. Relative frequency histogram of stem diameter for blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce.

CONCLUSIONS

The following deductions may be made from the results obtained in this study:

1. The pattern of inheritance of needle width, needle thickness, number of stomatal lines and needle sharpness suggests that they are generally polygenic in nature.

2. The inheritance of needle width, needle thickness, number of stomatal lines, and needle sharpness which distinguish blue spruce from Engelmann spruce is probably not maternally or paternally influenced.

3. Needle morphological traits may be inherited in blocks or groups, suggesting coherence of these characteristics.

4. The degree of overlap in range of diagnostic traits makes them unsuitable for definitive and rapid field determinations of Engelmann spruce x blue spruce hybridity.

5. Measurement of juvenile growth characters suggests that naturally occurring F1 hybrids which develop beyond early germination and seedling stages may be viable and healthy trees unencumbered by gross genetic abnormalities or obvious lack of vigor.

6. Given the above facts, it is apparent that natural F1 hybrids, although exceedingly rare, may exist.

CHAPTER IV

RECOMMENDATIONS FOR FUTURE STUDY

There remain many profound unanswered questions regarding species differenciation in the blue and Engelmann spruce complex. Although morphological differences have been the most studied aspect of these two species, many more subtle yet important traits have been overlooked or unmeasured, especially those traits which may have had the greatest impact in the speciation and isolation of the species *Picea pungens* Engelm.. The following represents an unprioritized partial list of studies that may add to further understanding of this topic.

1. Measure physiological attributes such as photosynthesis, resistance to solarization, growth, carbohydrate partitioning, localized enzymatic activity, or temporal levels of endogenous hormones in blue spruce, Engelmann spruce and their artificial hybrid Engelmann x blue spruce, at the Engelmann spruce altitudinal habitat.
2. Use the technique of DNA fingerprinting of Hillel, et.al.(1990) to determine the level of backcrossing in intermediate phenotypes discovered by Schaefer and Hanover (1990).
3. Investigate the genetic and physiological basis for incompatibility in blue and Engelmann spruce, comparing

factors such as pollen competition or chemical composition of the megastrobili exudate with that of the artificial hybrids.

4. Conduct a rangewide sampling of blue spruce and Engelmann spruce to compare morphological, physiological, and genetic attributes, while concurrently making more controlled crosses in an attempt to achieve the blue x Engelmann spruce reciprocal hybrid.
5. Conduct in vitro protoplast fusion experiments to identify particular genetic incompatibilities and to achieve novel hybrids for further study.

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APPENDICES

Appendix A. Buffers and reagents used in electrophoresis.

1. Extraction buffer for PGI (20 ml).

sodium phosphate	.114 g
EDTA	.007 g
ascorbic acid	.020 g
sodium bisulfite (meta)	.006 g
PVP - 40	1.000 g
beta-mercaptoethanol	.02 ml
D-fructose-6-phosphate	.020 g

2. Electrode buffer.

.029 M lithium Hydroxide
.190 M boric acid
10 N sodium hydroxide added to raise pH to 8.3

3. Gel buffer.

10 % electrode buffer
.05 M Trizma base
.0076 M citric acid
10 N sodium hydroxide added to raise pH to 8.3

4. Staining solution.

25	ml	Trizma HCl (pH 8.0) (24.22 g/l)
15	mg	D-fructose-6-phosphate
30	units	glucose-6-phosphate dehydrogenase
0.5	ml	Magnesium Chloride (10 mg/ml)
0.5	ml	NADP (10 mg/ml)
0.5	ml	NBT (10 mg/ml)
0.5	ml	PMS (1 mg/ml)

Appendix B. Materials used in morphological comparison

Species	Accession Number	Existing	Measured
Hybrids	6761 - 081	4	2
	- 082	4	2
	- 083	4	2
	- 098	1	1
	- 109	2	1
	- 111	2	2
	- 113	3	2
	- 115	3	2
	- 117	1	1
	- 118	6	4
	- 119	2	2
	- 130	1	1
	- 133	2	1
	- 135	1	1
	- 143	1	1
Totals	15	37	25

Species	Accession Number	Existing	Measured
P. engelmanii	6717 - 369	5	2
	- 370	4	2
	- 385	4	2
	- 386	5	1
	- 396	7	2
	- 398	4	1
	- 400	4	2
	- 402	2	1
	- 404	4	1
	- 406	5	3
	- 416	5	1
	- 420	5	2
	- 421	5	2
	- 428	5	1
	- 429	2	2
Totals	15	66	25

Appendix 2. (continued)

Species	Accession Number	Existing	Measured
P. pungens	6731 - 734	3	1
	- 738	5	1
	- 742	4	1
	- 746	4	1
	- 750	5	2
	- 754	5	1
	- 758	4	1
	- 762	3	1
	- 766	5	1
	- 770	4	2
	- 774	5	2
	- 778	5	1
	- 782	5	1
	- 786	4	1
	- 790	6	1
	- 794	4	1
	- 798	3	2
	- 802	5	1
	- 806	2	2
	- 810	5	1
Totals	20	86	25