The Putative Austrian x Red Pine Hybrid: A Test of Paternity Based on Allelic Variation at Enzyme-Specifying Loci[1])

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Summary

The authenticity of the most fully documented red pine hybrids, *Pinus nigra* (Austrian pine) × *P. resinosa* (red pine), was reassessed by a test of paternity based on allelic variation marked by enzyme variants. Seven enzymes were assayed by applying techniques of starch gel electrophoresis to female gametophyte and embryo tissues from seeds of controlled crosses between hybrids. Genetic analyses providing direct evidence of homology between genes from the two parental species indicate gene segregation at five loci, four of which appear to be linked. Data from embryos scored for three of these loci confirm that allele transmission through pollen parallels that found among female gametophytes. With this background, seven alleles at five loci contributed by the pollen parent of the hybrids were identified either by their absence from female gametophytes of the Austrian pine parent or because they are homozygous in some of the hybrids. In addition to these five loci, seven invariant zones of enzyme activity, assumed to be specified by seven homozygous loci, were assayed. For these loci, alleles contributed by the pollen parent are electrophoretically identical to those of the Austrian pine parent.

Of 14 alleles at 12 loci expected in the pollen parent of the hybrids, female gametophytes of red pine lacked eight. Moreover, red pine embryos with the nominal red pine parent of the hybrids as pollen parent lacked three of four alleles expected at three loci whose pollen transmission was documented in the hybrids. In contrast, a brief survey of female gametophytes of four other *Sylvestres* pines revealed pollen-parent alleles, often in substantial frequencies, at all twelve loci. We conclude that the pollen parent of these hybrids is not red pine, and that pollen contamination by some other *Sylvestres species* accounts for their paternity. It is suggested that other putative hybrids of red pine be accepted with caution.

Key words: interspecific hybrid, enzymes, gene segregation, *Pinus nigra, P. resinosa.*

Zusammenfassung

Die Echtheit der am vollständigsten belegten Hybriden der amerikanischen Rotkiefer, *Pinus nigra* (Schwarzkiefer) × *P. resinosa* (Amerikanische Rotkiefer) wurde durch eine Überprüfung der Vaterschaft über die Variation von durch Enzymvarianten markierten Allelen aufs neue bewertet. Durch Anwendung von Techniken der Stärkegel-Elektrophorese auf weibliche Gametophyten und Embryogewebe von Samen, die aus kontrollierten Kreuzungen zwischen Hybriden hervorgegangen waren, konnten sieben Enzyme untersucht werden. Genetische Analysen, die für eine Homologie zwischen Genen der beiden Elternarten einen direkten Anhaltspunkt liefern, weisen auf eine Genspaltung an fünf Loci hin, von denen vier gekoppelt zu sein scheinen. Daten, die von Embryos für drei dieser Loci gewonnen wurden, bestätigen, daß die Alleleübertragung durch Pollen, Parallelen zu den Ergebnissen hat, die bei den weiblichen Gametophyten gefunden wurden. Vor diesem Hintergrund wurde sieben Allele auf fünf Loci, die vom Pollenelter der Hybriden stammen, identifiziert und zwar entweder dadurch, weil sie am weiblichen Gametophyten des Schwarzkiefern-Elters nicht vorhanden waren oder weil sie in einigen der Hybriden homozygot waren. Zusätzlich zu diesen fünf Loci wurden sieben unveränderlich bleibende Zonen von Enzymaktivität untersucht, von welchen angenommen wird, daß sie von sieben homozygoten Loci markiert werden. Für diese Loci sind die Allele, die vom Pollenelter beigesteuert werden, elektrophoretisch identisch mit denen, die vom Schwarzkiefern-Elter stammen. Von den im Pollenelter der Hybriden erwarteten 14 Allelen auf 12 Loci waren acht bei den weiblichen Gametophyten der Rotkiefer nicht vorhanden. Weiter fehlten bei Embryonen der amerikanischen Rotkiefer, die denselben Baum wie die Hybriden als Pollenelter hatten, an den drei Loci drei von den vier erwarteten Allelen, von denen bei den Hybriden festgestellt wurde, daß sie durch den Pollen übertragen worden sind. Im Gegensatz dazu ergab eine kurze Überprüfung der weiblichen Gametophyten von vier anderen Kiefern der Subsektion *Sylvestres*, daß Allele des Pollenelters oft mit beträchtlichen Häufigkeiten auf allen zwölf Loci vorkamen. Wir schließen daraus, daß der Pollenelter dieser Hybriden nicht die amerikanische Rotkiefer ist und daß eine Verunreinigung der für die Kreuzung verwendeten Pollen, durch Pollen einiger anderer *Sylvestres* Arten, für die Vaterschaft verantwortlich ist. Es wird vorgeschlagen, andere vermeintliche Hybriden der amerikanischen Rotkiefer, mit Vorsicht als solche anzuerkennen.

Introduction

Red pine (*Pinus resinosa* Ait.) is not only one of the least variable of commercially important North American conifers (Fowler and Lester, 1970; Fowler and Morris, 1977), it is also one of the most difficult to hybridize with related taxa. Most attempts to hybridize red pine have concentrated on species of the subsection *Sylvestres (Lariciones)* of *Pinus*, a large, well-defined, predominately Eurasian group that includes red pine. In this paper we summarize the current status of interspecific hybridization of red pine, and reassess the authenticity of the first reported red pine hybrids: Austrian (*P. nigra* Arnold) × red pine. From a test of paternity based on allelic variation at enzyme-specifying loci, we conclude that red pine is not the pollen parent of these hybrids, and suggest that other putative hybrids of red pine be accepted with caution.

Many attempts have been made to hybridize red pine with other *Sylvestres* species, but few have been reported

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successful. WRIGHT and GABRIEL (1958) failed to obtain full seeds from crosses between red pine and eight other *Sylvestres* species. FOWLER (1964) used red pine as seed or pollen parent in crosses with 15 *Sylvestres* species or hybrids. Interspecific pollen mixtures were used to stimulate cone development in pollinations of more than 2700 strobili, but none of the resulting seedlings were red pine hybrids (FOWLER and LESTER, 1970). Unsuccessful attempts have also been made to cross red pine with *P. tropicalis* MORELET, the only other *Sylvestres* species native to the Western Hemisphere (HALL, KARNOSKY and FOWLER, 1976). ZUFA (1970, 1971) mixed irradiated red pine pollen with pollen from each of seven *Sylvestres* species or hybrids in crosses with red pine and obtained two *P. resinosa* × *nigra* seedlings and one *P. resinosa* × *densiflora* SIEB. and ZUCC. seedling. The identity of the hybrids was verified by two-dimensional paper chromatography of polyphenol extracts from needles (ZSUFFA, 1975). MOULALIS, BASSIOTIS and MITSOPOULOS (1976) obtained 16 putative hybrids of *P. nigra* × *resinosa* and 21 of *P. heldreichii* CHRIST × *resinosa*, but the authenticity of these hybrids remains to be established.

The oldest and most thoroughly described red pine hybrids are from a cross made in 1955 between an Austrian pine and a red pine growing in the arboretum of the Institute of Forest Genetics (IFG) at Placerville, California (CRITCHFIELD, 1963). Although most of the progeny from that cross were Austrian pines, six trees were identified as hybrids and described in detail by CRITCHFIELD. These trees were intermediate between Austrian and red pines in many respects, including conelet and cone morphology needle structure, and reproductive phenology. Most of the evidence was consistent with identification of the trees as Austrian × red pine hybrids, however, several anomalies were pointed out: (1) an attempt to repeat the cross with the same parent trees did not yield viable seeds; (2) an attempt to backcross one of the hybrids to red pine was unsuccessful; (3) "In one feature, the location of the principal resin canals in the leaves, some of the hybrids are unlike either parent species but resemble other pines in the *Lariciones* [*Sylvestres*] group." (CRITCHFIELD, 1963).

With one exception, all subsequent attempts to repeat the cross between Austrian and red pines and to backcross the hybrids to red pine have been unsuccessful. Reciprocal crosses between the original parent trees, involving pollination of 109 female strobili in two seasons, produced a single putative hybrid. (This tree is just reaching reproductive maturity and could not be include in this study.) Other crosses between the two species, involving 123 female strobili, produced no germinable seeds. Attempts to backcross the hybrids to their red pine parent and to other red pines, using four hybrids as female parents in pollinations of 148 strobili in four seasons, also failed. In contrast, all backcrosses of the hybrids to Austrian pines were successful, and crosses with their maternal parent averaged 4.4 germinable seeds per cone.

Although the trees are definitely hybrids and not Austrian pines (CRITCHFIELD, 1963), the above observations have led us to question their red pine parentage. To test the authenticity of the hybrids, we employed techniques of gel electrophoresis to analyze allelic variation at individual loci. The strategy adopted is to partition the diploid genotype of each hybrid into two haploid genotypes: one from the Austrian pine parent and the other from the pollen parent in question. For homozygous loci, the two haploid genotypes are identical. But, for loci that segregate in the hybrids, it follows that the allele not present in the seed parent must have been contributed by the pollen parent. Applying this rationale to a number of segregating loci makes it possible to deduce for each hybrid the haploid genotype of the gamete received from the pollen parent. It is thus possible to test the paternity of the hybrids by surveying alternative pollen-parent species for alleles required to reconstruct these haploid genotypes.

The validity of this approach depends on an unambiguous identification of allelic variation and evidence of homology among loci from different species. Allelic variation is assessed by a direct analysis of gene segregation, obtained by testing for a one-to-one ratio of enzyme variants among haploid female gametophytes of an individual tree. Since we are concerned with gene transmission through pollen, we also analyze gene segregation among male gametophytes by assaying embryo and female gametophyte tissues from seeds of controlled crosses between hybrids. These analyses provide direct evidence of homology between genes from the two parental species that segregate in the hybrids.

Materials and Methods

Seed Collections

Control- or wind-pollinated seeds were provided by the IFG, except for one collection from a red pine (ST 801) growing at Fredericton, New Brunswick, and included in an earlier study (FOWLER and MORRIS, 1977). Arboretum tree numbers and species codes are those of the IFG.

P. resinosa: Re V26 × Re V29. Re V29, the nominal pollen parent of the Austrian × red pine hybrids described by CRITCHFIELD (1963), has since died. The seed parent of this cross, Re V26, is from the same unspecified Maine source as Re V29.

P. *nigra var. austriaca:* Ni N7 × wind. Ni N7 is the seed parent of the hybrids.

P. nigra var. *austriaca* × *resinosa*: Seeds from controlled crosses among three of the four hybrids (NiRe 11, 12, 13, 14) were used for genetic analyses: NiRe 11 × NiRe 12, NiRe 12 × NiRe 11, NiRe 12 × NiRe 13. We also assayed small numbers of seeds from NiRe 13 × NiRe 11 and NiRe 14 × wind.

In 1955, the year in which the Austrian × red pine cross was made, pollen was collected from 12 other *Sylvestres* species, all of which were potential pollen contaminants in the laboratory. Extrapolating from characters of the hybrids (CRITCHFIELD, 1963), we looked for an alternative pollen parent with smaller cones than *P. nigra* and needles as long as or longer than those of *P. nigra,* with some or all of the principal resin canals external and adjacent to the abaxial (rounded) face of the needle. Several candidates were eliminated because their principal resin canals are either medial, like those of *P. nigra (P. thunbergiana, P. heldreichii. P. luchuensis, P. pinaster)* or external but adjacent to the flat face of the needle, like those of *P. resinosa (P. halepensis).* Three species were eliminated because of their short needles (*P. mugo, P. sylvestris)* or large cones *(P. yunnanensis).* We analyzed female gametophytes of the four remaining species, all native to eastern Asia:

P. densiflora: Equal numbers of seeds from three collections from Japan (IFG Lots AA, AB, AC).

P. tabulaeformis: One collection from China (Lot J).

P. taiwanensis: Equal numbers of seeds from three collections from Taiwan (Lots F, G, H).

P. massoniana: Equal numbers of seeds from three collections from China (Lots J, N, O).

Laboratory Procedures

Seeds were stratified and germinated on moist filter paper in a germination chamber. Germinated seeds with radicles 1 to 10 mm long were assayed. The elongating embryo and its associated female gametophyte were individually macerated in two or three drops of .2M phosphate buffer, pH 7.5. Procedures of starch gel electro-

phoresis followed those described previously (FOWLER and MORRIS, 1977). As before, enzymes were separated on two different starch gels, but for this study the tris-citrate-lithium-borate buffer of gel A was that of SCANDALIOS (1960).

The following enzyme activities, identified by their enzyme commission numbers, were assayed: *gel A*--leucine aminopeptidase (LAP; EC 3.4.11.1), alcohol dehydrogenase (ADH; EC 1.1.1.1), phosphoglucomutase (PGM; EC 2.7.5.1); *gel B*--acid phosphatase (ACPH; EC 3.1.3.2), glutamate dehydrogenase (GDH; EC 1.4.1.2), glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1), glucose-6-phosphate dehydrogenase (G-6PD; EC 1.1.1.49).

Alcohol dehydrogenase was assayed by the method of SCANDALIOS (1969). Phosphoglucomutase and glucose-6-phosphate dehydrogenase were assayed by the methods of BREWER (1970) with 10 mg of NADP added during preparation of each B gel to increase staining of G-6PD. Other enzyme assays were unchanged.

Genetic Analysis

A complete analysis of the gametic transmission of alleles marked by enzyme variants is obtained by assaying embryo and female gametophyte tissue from seeds of controlled crosses among hybrids. Assuming the band mobility that occurs in both haploid (female gametophyte) and diploid (embryo) tissues from the same seed represents an allele transmitted by the female gamete, it follows that the remaining allele present in embryo tissue was contributed by the male gamete.

Restricting attention to a single gamete pool, assayed directly as female gametophytes or inferred from embryos, the gametes of a putative double heterozygote can be arranged in a factorial table as follows:

		Enzyme A	
		A_1	A_2
Enzyme B	B_1	n_{11}	n_{12}
	B_2	n_{21}	n_{22}

where the n_{ij}'s are observed numbers of pairs of enzyme phenotypes. Random gene segregation is tested with a χ^2 statistic for goodness-of-fit of the marginal sums to a one-to-one ratio. For each enzyme, the null hypothesis $p = 1/2$ is tested against a two-sided alternative with one degree of freedom. The remaining degree of freedom is used to assess linkage by a χ^2 test for independence with null hypotheses $P(A_i \cap B_j) = P(A_i) \cdot P(B_j)$, i, j = 1,2. This test is independent of tests for random gene segregation. If the parental phenotypes inferred in this study are denoted A_1B_1 and A_2B_2, then the appropriate alternative hypotheses are one-sided: $P(A_i \cap B_i) > P(A_i) \cdot P(B_i)$ and $P(A_i \cap B_j) < P(A_i) \cdot P(B_j)$, $i = j$. A one-sided test is obtained by noting that a χ^2 with one degree of freedom is the square of a standard normal, therefore, $\sqrt{\chi^2}$ is compared to the standard normal curve when observed numbers differ from expected numbers in the direction specified by alternative hypotheses. Different gamete samples are tested by χ^2 for homogeneity and, in the absence of interaction, gamete totals are subjected to the single degree of freedom tests described above.

Results

Genetic Interpretation of Enzyme Phenotypes

Preliminary genetic hypotheses were based on assays of female gametophytes from two hybrids, NiRe 11 and NiRe 12.

GDH and PGM: Both enzymes exhibit a single band of activity and no variation in mobility. Thus, each enzyme is assumed to be specified by at least one gene, designated *Gdh1* and *Pgm1*.

LAP: Two bands of LAP activity are present, neither of which varies in mobility. Although no direct evidence for a one-band one-gene relationship is available from this study, we adopt such a relationship from the work of NIKOLIC and BERGMANN (1974), and designate two loci, *Lap1* and *Lap2*, in order of decreasing mobility.

G-6PD (*Figure 1*): Three G-6PD isozymes are present. The slowest band is invariant, but the intermediate and fast-migrating bands vary among female gametophytes of both trees. Since the slowest band is insensitive to variation in the other two bands, it is assumed to represent a distinct gene, *G-6pd2*. Phenotypes of the two variable bands are completely associated: one phenotype exhibits a dark-staining fast band and light-staining intermediate band, and the other phenotype shows a light-staining fast band and dark-staining intermediate band. Either a single locus is segregating two enzyme forms or two tightly linked loci are segregating as a unit. Since no putative recombinants were recovered from 172 female gametophytes,

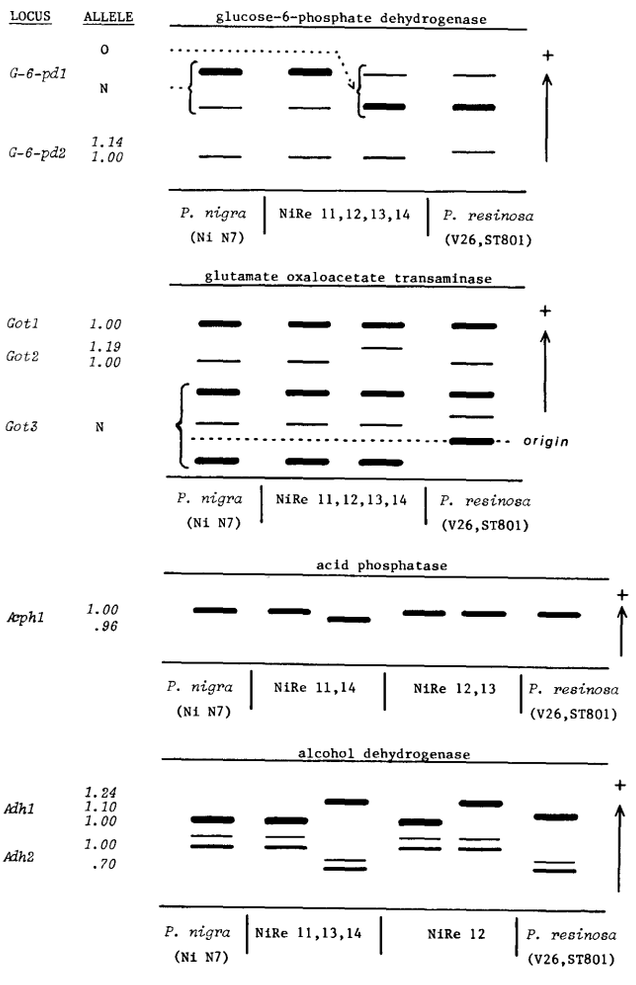

Figure 1. — Diagrams and genetic interpretations of glucose-6-phosphate dehydrogenase, glutamate oxaloacetate transaminase, acid phosphatase and alcohol dehydrogenase phenotypes of female gametophytes from *P. nigra* (Ni N7), NiRe 11, NiRe 12, NiRe 13, NiRe 14, and *P. resinosa* (Re V26, ST 801).

one gene, G-*6pd1*, is assumed to determine both the intermediate and fast-migrating bands.

GOT *(Figure 1)*: Female gametophytes of both trees exhibit two five-banded phenotypes that differ only in the relative mobility of the second fastest anodal band of activity. Since variation in this zone does not affect other band mobilities, there must be more than one gene specifying GOT activity. The position of the variable zone between invariant bands suggests that the fastest anodal band is determined by a different gene than those bands nearest the origin.

Evidence supporting this interpretation is found in the Austrian pine parent, Ni N7, where two phenotypes are observed in which the three bands nearest the origin shift mobility in register. The lack of variation in the fastest and second fastest anodal bands among female gametophytes of this tree is consistent with the hypothesis that these two zones of activity are determined by genes distinct from the gene(s) specifying bands nearest the origin. From these observations, we tentatively adopt an hypothesis of three GOT loci, *Got1, Got2* and *Got3*.

ADH *(Figure 1)*: Three zones of activity are present. The most anodal zone varies widely in staining intensity and often is not visible. Since no obvious genetic interpretation is suggested, this zone is excluded from further consideration and is omitted from *Figure 1*. Of the other two zones, the faster migrating, denser staining one exhibits two mobilities in female gametophytes of both trees. The zone nearest the origin, consisting of two light and unequally staining bands, is invariant among female gametophytes of NiRe 12, but both bands shift mobility among female gametophytes of NiRe 11. Our data alone do not discriminate between a single-locus model and models involving two or three tightly linked loci. However, from the work of Rudin and Ekberg (1978) on *P. sylvestris*, we assume two tightly linked loci and designate *Adh1* as the gene responsible for the dark fast band and *Adh2* as the gene specifying the much lighter staining doublet.

ACPH *(Figure 1)*: ACPH exhibits multiple zones of activity but only the dark-staining, fastest migrating band admits to a simple genetic interpretation. Female gametophytes of NiRe 12 show a single band mobility while those of NiRe 11 exhibit two band mobilities. Therefore, a single locus, *Acph1, is* inferred to specify this zone.

Female gametophytes of NiRe 13 and NiRe 14 were also assayed. Phenotypes of nine female gametophytes from NiRe 14 and four from NiRe 13 were identical to those of NiRe 11, except that only one ACPH band mobility was observed in female gametophytes of NiRe 13. Thus phenotypes of the four hybrids differ in only two of the enzymes studied: *Acph1* exhibits two band mobilities in NiRe 11 and NiRe 14 but only one in NiRe 12 and NiRe13, and *Adh2* varies in all hybrids except NiRe 12.

Enzyme Phenotypes of the Maternal Parent

The maternal parent of the hybrids, Ni N7, is invariant for all enzymes studied, except GOT. As noted above, two triple-banded *Got3* phenotypes occur in female gametophytes of Ni N7, but only the phenotype illustrated in *Figure 1* is found in the hybrids. The three invariant enzymes of the hybrids, GDH, PGM and LAP, exhibit the same band mobilities in female gametophytes of Ni N7. Among variable enzymes, one of two phenotypes present in the hybrids is also present in female gametophytes of Ni N7 *(Figure 1)*.

Genetic Analysis of Enzyme Variants

Gametic distributions of enzyme variants marking five putative loci, *G-6pd1, Got2, Adh2* and *Acph1*, were analyzed in seeds from three crosses: NiRe 11 × NiRe 12, NiRe 12 × NiRe 11 and NiRe 12 × NiRe 13. G-6PD phenotypes could not be scored in embryos and were analyzed only in

Table 1. — Segregation Analysis of Four Loci

	Locus							
	G-6pd1		Got2		Adh1		Acph1	
Gamete Origin	Allele N	Allele O	Allele 1.00	Allele 1.19	Allele 1.00	Allele 1.24	Allele 1.00	Allele .96
Female Gametophytes								
NiRe 11	35	39	45	44	33	43	29 *	51
NiRe 12 (12 X 11)	35	34	36	42	29	41	xx	xx
NiRe 12 (12 X 13)	14	15	17	24	12 **	29	xx	xx
Male Gametophytes								
NiRe 11	--	--	41	37	33	37	29	41
NiRe 12	--	--	49	40	29	41	xx	xx
NiRe 13	--	--	24	17	24	17	xx	xx
Total	84	88	212	204	160 *	208	58 **	92

xx homozygous for allele 1.00
-- phenotypes not resolved in embryo tissue
Homogeneity χ^2: *G-6pd1*, $\chi^2_2 = .17$ ($p > .5$); *Got2*, $\chi^2_5 = 3.88$ ($p > .5$); *Adh1*, $\chi^2_5 = 7.77$ ($p > .1$); *Acph1*, $\chi^2_1 = .42$ ($p > .5$).

* = $p < .025$
** = $p < .010$

Table 2. — Linkage Analysis of Three Loci in Male and Female Gametophytes

Loci	Gamete Origin		Two-Locus Genotypes: Parental		Non-Parental		$\sqrt{\chi^2}$	Probability	Recombination Fraction r $\pm\sqrt{\frac{r(1-r)}{N}}$
Acph1/Got2			1.00/1.00	.96/1.19	1.00/1.19	.96/1.00			
	NiRe 11	Female	14	26	15	25	*		
		Male	17	22	12	19	1.01	$p < .16$	
		Total	31	48	27	44	.67	$p < .25$	
Acph1/Adh1			1.00/1.00	.96/1.24	1.00/1.24	.96/1.00			
	NiRe 11	Female	14	29	9	15	2.10	$p < .02$	
		Male	13	21	12	16	.68	$p < .25$	
		Total	27	50	21	31	1.98	$p < .02$	.403 ± .043
Adh1/Got2			1.00/1.00	1.24/1.19	1.00/1.19	1.24/1.00			
	NiRe 11	Female	23	29	10	14	3.21	$p < .001$	
		Male	21	21	12	16	1.71	$p < .04$	
	NiRe 12	Female (12 X 11)	19	28	10	13	2.80	$p < .003$	
		Female (12 X 13)	6	18	6	11	.71	$p < .24$	
		Male	22	25	7	16	3.05	$p < .001$	
	NiRe 13	Male	16	9	8	8	1.26	$p < .10$	
		Total	107	130	53	78	5.59	$p \sim .000$	.356 ± .025

Homogeneity χ^2: *Acph1/Got2*, $\chi^2_3 = 1.12$ ($p > .75$); *Acph1/Adh1*, $\chi^2_3 = 1.59$ ($p > .50$); *Adh1/Got2*, $\chi^2_{15} = 12.21$ ($p > .50$).
* Observed numbers differ from expected numbers contrary to alternative hypotheses.

female gametophytes. Variants assigned to *Adh2* were also limited to female gametophyte analysis, but these data are not presented explicitly since, due to a complete association of band mobilities in female gametophytes of NiRe 11, they are a subset of *Adh1* data.

Table 1 presents the numbers of gametes scored for alternative phenotypes of four enzymes. The numbers of female gametophytes exhibiting alternative phenotypes of G-6PD correspond closely to a one-to-one ratio. The two band mobilities of GOT are also equally frequent, not only in female gametophytes but among male gametes as well. In contrast, the numbers of gametes transmitting different band mobilities of ADH and of ACPH depart from equality to a degree that is unlikely a chance result of finite sampling.

Table 2 gives the results of linkage analysis in male and female gametes. Phenotypes of ACPH and GOT are independently associated, but the joint phenotype distributions of ACPH-ADH and ADH-GOT exhibit a greater frequency of parental phenotypes than is expected on an hypothesis of independence. The pattern of association indicates three loosely linked loci in the order: *Got2* - *Adh1* - *Acph1*. By hypothesis, *Adh2* is tightly linked to *Adh1*, but their relative order is unknown. A similar analysis, limited to female gametophytes, shows that *G-6pd1* assorts independently of this linkage group, but the details are omitted.

Since linkage tests are independent of segregation tests, the pattern of nonrandom association among GOT, ADH and ACPH phenotypes can be taken as evidence for their genetic control, and the homogeneity of both segregation and linkage data over male and female gametes is consistent with this interpretation. Therefore, in spite of the loose fit of the segregation data for ADH and ACPH to a simple Mendelian model, we conclude that the variation in four enzymes is determined by allelic variation at five loci, *G-6pd1*, *Got2*, *Adh1*, *Adh2* and *Acph1*.

Pollen Genotypes of the Paternal Parent

Five alleles segregate in the hybrids but do not occur in the Austrian pine parent, Ni N7: *G-6pd1*^{0}, *Got2*$^{1.19}$, *Adh1*$^{1.24}$, *Adh2*$^{.70}$ and *Acph1*$^{.96}$. All of these alleles are expected in the pollen parent. Two other alleles, *Adh2*$^{1.00}$ and *Acph1*$^{1.00}$, are homozygous in some of the hybrids, and therefore must also be present in the pollen parent (see *Figure 1*).

Enzyme Phenotypes of Red Pine

Female Gametophytes. Since female gametophytes of Re V29, the nominal red pine parent of the hybrids, were not available, we compared female gametophytes of Re V26, a red pine from the same geographic origin as Re V29, to those of the hybrids and Ni N7 on the assumption that female gametophytes of Re V26 and Re V29 exhibit the same phenotypes. This assumption is based on an earlier study of red pine (Fowler and Morris, 1977) in which no polymorphism was detected in four of the enzymes (LAP, G-6PD, GOT, ACPH) studied here, and is supported by the observation that female gametophytes of Re V26 and ST 801, the red pine standard of our previous study, exhibit identical phenotypes for all seven enzymes of this study.

Of seven alleles at five loci expected in the pollen parent

of the hybrids, female gametophytes of Re V26 exhibit three: *G-6pd1*^{0}, *Adh2*$^{.70}$ and *Acph1*$^{1.00}$, but lack four: *Got2*$^{1.19}$, *Adh1*$^{1.24}$, *Adh2*$^{1.00}$ and *Acph1*$^{.96}$ *(Figure 1).* Among invariant enzymes of the hybrids, Re V26 shows the same phenotype at three loci: *Pgm1, Lap2* and *Got1,* but exhibits a different one at four loci: *Gdh1, Lap1, G-6pd2* and *Got 3.* All together, the red pine phenotype is consistent with that expected for the pollen parent at four loci, lacks one of two alleles at each of two loci, and differs completely at the remaining six loci. In view of the low level of enzyme polymorphism in red pine (FOWLER and MORRIS, 1977), these results suggest that red pine is not the pollen parent, but the possibility remains that Re V29 is atypical among red pines and is in fact the true pollen parent.

Embryos. To directly assess the possibility that pollen from Re V29 transmits alleles deduced to have originated from the actual pollen parent of the hybrids, we have assayed red pine embryos that have Re V29 as pollen parent. Embryos from the cross Re V26 × Re V29 were scored for the presence of three pollen-parent alleles whose transmission through pollen was established by genetic analysis of crosses among the hybrids. Band mobilities marking alleles *Got2*$^{1.19}$, *Adh1*$^{1.24}$ and *Acph1*$^{.96}$, all of which are expected in the actual pollen parent, were not found among ten embryos from this cross while those that did occur were the same as found in female gametophytes of Re V26.

This observation virtually rules out Re V29 as the pollen parent of the hybrids, for if pollen from Re V29 were segregating alleles *Got2*$^{1.19}$, *Adh1*$^{1.24}$ and *Acph1*$^{.96}$, the probability of not recovering a single one of them among ten embryos would be on the order of 10^{-8} or less, depending upon the linkage configuration involved. Apparently pollen from Re V29 does not transmit these three alleles, but rather contains the same three as Re V26. We therefore conclude that the pollen parent of the hybrids is neither Re V29 nor any red pine.

Alternative Pollen Parents

Female gametophytes of four species were assayed: *P. densiflora, P. taiwanensis, P. massoniana* and *P. tabulaeformis. Table 3* lists, for each of twelve loci, the pollen-parent allele and the frequency of that allele among female gametophytes of the four candidates.

Alleles required to reconstruct the haploid genotypes of pollen responsible for the hybrids are present in substantial frequencies in female gametophytes of *P. densiflora* and P. *taiwanensis.* The same is true for P. *tabulaeformis,* with the exception of *Gdh1* for which no data are available. *P. massoniana,* however, appears less likely than the other species to contain the required haploid genotypes.

From this limited survey of alternative pollen parents, we conclude that the red pine pollen used in the 1955 cross from which the hybrids originated was contaminated by

Table 3. — Frequencies of Pollen-Parent Alleles in Female Gametophytes of Four Pine Species.

		Species			
Locus	Allele	P. densiflora	P. taiwanensis	P. massoniana	P. tabulaeformis
Gdh1	1.00	.94 (18)	1.00 (18)	.83 (18)	ND
Pgm1	1.00	.93 (15)	.85 (13)	.08 (12)	1.00 (12)
Lap1	1.00	.90 (38)	.61 (38)	.00 (34)	.88 (33)
Lap2	1.00	.92 (38)	.95 (38)	.91 (34)	.91 (33)
G-6pd1	0	.86 (21)	.71 (21)	1.00 (21)	1.00 (32)
G-6pd2	1.00	.14 (21)	.10 (21)	.05 (21)	.28 (32)
Got1	1.00	1.00 (22)	1.00 (21)	1.00 (21)	.70 (32)
Got2	1.19	.95 (22)	.76 (21)	.90 (21)	.94 (32)
Got3	N	.91 (22)	.71 (21)	1.00 (21)	.63 (32)
Acph1	1.00 .96	.55 .18 (22)	.71 .10 (21)	.86 .05 (21)	.38 .12 (31)
Adh1	1.24	1.00 (21)	1.00 (22)	1.00 (21)	.97 (33)
Adh2	1.00 .70	.29 .71 (21)	.18 .50 (22)	.00 .43 (21)	.21 .66 (33)

The number of female gametophytes assayed are in parentheses. ND - no data.

pollen from some other species of the *Sylvestres* group. Those species surveyed, however, are so similar in the frequencies of pollen-parent alleles that only *P. massoniana* can be considered unlikely as a candidate for the actual pollen parent. Indeed, our brief survey does not exclude other *Sylvestres* pines, nor does it rule out the possibility that pollen from more than one species was involved in the paternity of the hybrids.

Discussion

Accepting the hypothesis that variants of ACPH and ADH reflect allelic variation requires that some mechanism altering normal gene segregation be operating at or following meiosis. There are a number of such mechanisms, including preferential segregation, gametophytic viability selection, pollen competition, and some forms of zygotic selection. Of these, only pollen competition is ruled out by the segregation data of *Table 1* since that would lead to skewed segregation ratios among male but not among female gametes. Although no distinction between the remaining hypotheses is possible with the data presented here, we note that the phenomenon of segregation distortion has been reported in other conifers (Lundkvist, 1974; Rudin, 1975, 1977; Rudin and Ekberg, 1978) and is well documented in angiosperms, especially for interspecific hybrids (Grant, 1975).

For the five loci subjected to genetic analysis, interspecific comparisons of phenotypes rest on direct evidence of homology, but for homozygous loci no such evidence is available. However, if we interpret enzymes that do not vary among female gametophytes of the hybrids as resulting from genes that are homozygous, it follows that homologous loci in male and female parents must contain alleles that specify electrophoretically identical phenotypes. Even though assignment of individual loci to particular enzyme bands may in some cases be incorrect, we are nevertheless led to predict that invariant phenotypes of the hybrids, all of which occur in the seed parent, should also be found in the pollen parent. It is on this basis that we have included invariant enzymes as criteria for testing the paternity of the hybrids.

In comparing phenotypes of candidate species to those expected from the actual pollen parent, we have implicitly assumed that identical phenotypes reflect identical genotypes. But not all allelic differences are detectable with electrophoresis. As a result, phenotypes indistinguishable from those of the actual pollen parent may be specified by alleles other than those present in the hybrids, and the extent to which such alleles occur in candidate species limits the possibility of correctly singling out the true pollen parent.

Apart from the inherent limitations of phenotypic analysis, of greater significance in limiting the ability to identify the true pollen parent is the sampling of female gametophytes of the Asian pines. In the absence of seeds from trees that provided pollen during the 1955 season, we have assayed seeds from the native range of these species. As a consequence, identification of the pollen-parent species depends upon the extent of allele frequency differentiation within and between species rather than discrete allelic differences between individuals, as would be the case were seeds from arboretum trees available. Based on our small survey, the Asian pines differ little in the frequencies of alleles specifying pollen-parent phenotypes, therefore on these data alone it is not possible to determine which, if any, of these species is the pollen parent.

Wright and Gabriel (1958), in their comprehensive exploration of species hybridization in subsection *Sylvestres*, present data that make it possible to quantify the efficiency with which *P. nigra* can be crossed with each of the four Asian pines included in this study. Expressing "crossability" as the ratio of percent sound seed from an interspecific cross to percent sound seed from a conspecific cross, *P. densiflora* ranks highest with a value of .25, followed by *P. taiwanensis* (.15) and *P. tabulaeformis* ($< .02$). Wright and Gabriel obtained no full seeds in small scale crosses between *P. nigra* and *P. massoniana*, and two attempts to cross these two species at the IFG were also unsuccessful. These limited data reinforce our tentative conclusion based on enzyme data that *P. massoniana* is not the pollen parent of the hybrids, but they are not decisive in ruling out other candidates. Because of its higher crossability, *P. densiflora* appears to be the most likely candidate for pollen parent, but when mixtures of *P. nigra* and *P. densiflora* pollen are used to pollinate *P. nigra*, hybrids are substantially underrepresented in the progeny (Tobolski and Conkle, 1977).

In spite of numerous attempts by tree breeders, only a few interspecific hybrids of red pine have been reported. Of these, as the results of this study indicate, the oldest and most thoroughly described are not red pine hybrids, but rather hybrids between *P. nigra* and another (unknown) species. In view of this negative evidence and the limited documentation of other red pine hybrids, we suggest that reports of interspecific hybrids with red pine as a parent be accepted with caution.

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Genetic differentiation in ponderosa pine along a steep elevational transect

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Summary

Data are reported for 7 enzyme polymorphisms in ponderosa pine, *Pinus ponderosa* LAWS. var. *scopulorum* ENGELM., in 3 ecologically different localities along a steep elevational transect in the Front Range of Colorado. The highest and lowest sites of the transect differ by almost 1000 m. in elevation, but they are only 3 km apart. Heterozygote excesses associated with slope aspect are found at a peroxidase locus, and clinal differentiation is evident at a phosphoglucomutase locus. Linkage disequilibrium, a measure of non-random association of genotypes at separate loci, is found at each of the localities.

Key words: ponderosa pine, genetic differentiation, linkage disequilibrium allozymes, elevational transect.

Zusammenfassung

Es wird über 7 Enzympolymorphismen bei *Pinus ponderosa* LAWS var. *scopulorum* ENGELM. an drei ökologisch verschiedenen Orten berichtet. Diese liegen entlang eines Höhengradienten im Front Range von Colorado. Der maximale Höhenunterschied zwischen zwei Standorten beträgt 1000 m, wobei diese nur 3 km voneinander entfernt sind. Bei einem Peroxidase-Locus wurde ein Zusammenhang zwischen Heterozygotie und Hanglage festgestellt, eine klinale Variation wurde bei einem Phosphoglucomutase-Locus deutlich. Kopplungsungleichgewichte, ein Maß für die nicht zufallsmäßige Zusammensetzung von Genotypen an einzelnen Loci wurden für jeden der Versuchsorte festgestellt.

Introduction

Traditional views of the relative influence of gene flow and environmentally specific selection upon genetic differentiation and speciation led many biologists to assume that there would be little differentiation within continuous populations (MAYR, 1963). In spite of a growing body of evidence demonstrating that genetic differentiation does occur over short distances in many plants (JAIN and BRADSHAW, 1966; EHRLICH and RAVEN, 1969; GRANT and MITTON, 1977; MITTON *et al.*, 1977; WHITE, 1978) there still remains considerable skepticism that differentiation can occur within continuous stands of long-lived woody perennials (ENDLER, 1977). On the contrary, we have documented considerable genetic differentiation both between closely adjacent stands associated with ecological parameters such as slope aspect (MITTON *et al.*, 1977) as well as within stands associated with family structure (LINHART *et al.* 1980). Although the role of natural selection in molding population structure is strongly suggested by most of these studies, more empirical evidence is needed before the relative contributions of natural selection, migration, family structure, and stochastic forces can be assessed.

Presented here are results from a study of 7 protein polymorphisms in sample localities along an elevational gradient near Boulder, Colorado. A steep transect has been studied to maximize both the possibility of gene flow and the ecological and environmental distinction between the sample localities. Analyses are presented of both single gene polymorphisms and the joint distributions of pairs of polymorphic loci.

Materials and methods

Description of sites

Ponderosa pine, *Pinus ponderosa* LAWS. vars *scopulorum*, is a long-lived, wind-pollinated temperate forest tree. It forms extensive, continuous stands in the Montane and Upper Montane region between 1829—2743 meters in the Colorado Rocky Mountains. On south-facing slopes it occurs in pure, open stands with a lush understory of grasses and herbs. On steep north-facing slopes, it is co-dominant with Douglas-fir, *Pseudotsuga menziesii*, forming dense stands through which little sunlight penetrates to the forest floor (MARR, 1967).

The transect studied here runs from east to west over a horizontal distance of 3 kilometers and 811 meters of elevational gain. The lowermost site (elev. 1767 m) is a savannah-like pure stand of relatively large diameter, short pines that are actively colonizing the grasslands of the Great Plains (ROBBINS and DODDS, 1908; ROACH, 1948). The slope is almost flat; trees are widely spaced and have an open-grown appearance. East of these trees, conifers are absent for hundreds of miles expect for a few isolated stands. The highest elevation site (elev. 2579) m.), by contrast, consists of a