

GEOGRAPHIC PATTERN OF GENETIC DIVERSITY IN *PINUS RESINOSA*: CONTACT ZONE BETWEEN DESCENDANTS OF GLACIAL REFUGIA¹

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Although red pine (*Pinus resinosa*) generally has low or completely lacks variation for molecular markers, some variation is observed for chloroplast microsatellites (cpSSRs). We sampled and examined 10 cpSSRs for 19 populations. Analysis of these populations plus 10 previously studied populations shows that the geographic distribution of genetic diversity over the range of *P. resinosa* is markedly nonuniform. Although the pattern exhibits little isolation by distance, there is a region centered in northeastern New England where populations contain much greater chloroplast haplotype diversity than elsewhere. This area is band-shaped, with the longer axis nearly parallel with latitude, and very sharply delineated. The area of high diversity was buried by the Laurentide ice sheet. The geographic pattern indicates that *P. resinosa* is not at equilibrium, and the species has had a more complex postglacial history than typically purported for forest trees in eastern North America. The results suggest that the area of high diversity is a stable transition zone between descendants of two distinct refugia, one in the southern Appalachians and another near the North Atlantic coastline of the Wisconsinian glacial period. Plausible explanations are given that selection between two lineages, along latitudinal zones, may have maintained the transition zone.

Key words: admixture; chloroplast DNA; microsatellite; *Pinus resinosa*; population genetics; refugia.

Red pine (*Pinus resinosa* Ait.) holds a unique position among North American pines in that it has both very low amounts of genetic diversity and a wide geographic range. For example, a number of studies have been unsuccessful in finding any polymorphism for allozymes (Fowler and Morris, 1977; Simon et al., 1986; Allendorf et al., 1982; Mosseler et al., 1991), in stark contrast to the rather high levels of polymorphism found in nearly every other pine. Among studied gymnosperms (mostly conifers) an average of 71% of allozyme loci are polymorphic at the species level (Hamrick and Godt, 1990). Hamrick et al. (1992) examined 93 genetic studies of pine species and found an average heterozygosity of 0.157 for allozymes, whereas it is effectively zero in red pine. Mosseler and colleagues (1992) found virtual monomorphism for a number of randomly amplified polymorphic DNA (RAPD) markers. However, a few variants were observed in Newfoundland, and these were verified by restriction fragment length polymorphism (RFLP)-RAPD analyses (DeVerno and Mosseler, 1997).

Despite the fact that red pine is by most standards depauperate in genetic variation, polymorphism for chloroplast microsatellite or simple sequence repeat (cpSSR) loci has been found (Echt et al., 1998). This finding does not necessarily conflict with other studies, because cpSSRs may mutate at greater rates than do most loci (e.g., Di Rienzo et al., 1994; Provan et al., 1999). The amount of genetic diversity of cpSSR loci in red pine is small compared to that in other pines (e.g., Powell et al., 1995; Vendramin et al., 1996). Clearly, red pine has reduced variation for molecular markers, despite there being millions of individuals spread over its present range across

the northcentral and northeastern United States and southcentral and southeastern Canada (Fig. 1).

While numerous studies by forest geneticists and tree breeders have emphasized red pine's paucity of morphological variation in comparison to other forest trees, these studies do find some genetically based morphological variation. Typically, about 10% of morphological variation is genetically determined by source population, in common garden experiments known as provenance tests (reviewed by Fowler and Lester, 1970; Wright et al., 1972). Traits include survival and growth rates (Wright et al., 1972; Yao, 1974; Guries and Ager, 1980), phenology, wood quality (Fowler and Lester, 1970), and branching characteristics (Mosseler et al., 1992). Generally, many traits in pines and other forest trees show concordant geographical patterns of genetic variation. Significantly, red pine shares these patterns, even though the overall amounts of genetic differentiation are smaller.

Prominent among geographic patterns in red pine are traits associated with latitude. For example, Vaartaja (1962) found significant genetic differences in photoperiodic seedling growth response, such that under controlled short day schedules plants from northern populations cease growing and set buds earlier. Such a pattern of dormancy is generally considered a geographic adaptation in forest trees for avoiding early fall frost damage. Similarly, there are significant differences in the timing of flowering; for example, northerly red pine forests undergo anthesis up to 3–4 wk later. Frost damage is a significant problem for red pine, and the average annual frost-free period varies widely, from only 60 d up to 180 d, across the present day native range (Fowler and Lester, 1970). Dhir (1973) also found source differences in seedling shoot traits, and some of these traits are correlated with latitude. Northern trees appear to produce smaller seed in provenance tests and also suffer less frost damage (Fowler and Lester, 1970). In addition, individuals of a small isolated population in Illinois (Fig. 1) produce white pollen (Brenneman, 1956), a unique condition in red pine, and a rare, usually recessive

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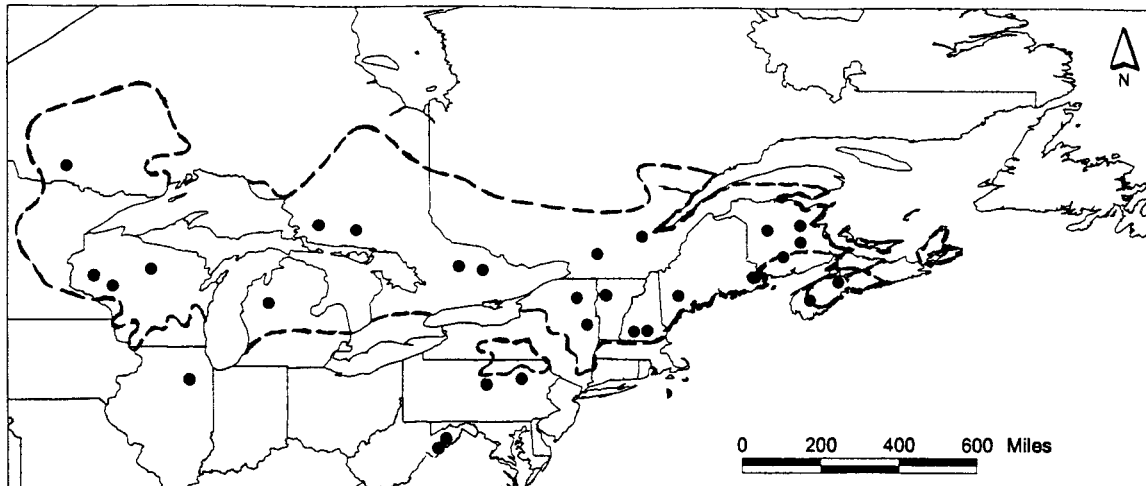


Fig. 1. Range map. Locations of sampled populations are indicated by closed circles, including disjunct populations, outside the main range, which is patterned after Little (1971).

genetic condition in pines generally (Johnson and Critchfield, 1974).

Despite geographic genetic variation for important morphological traits, the paucity of variation for genetic markers in red pine indicates that it has suffered severe bottleneck(s) in population size. This also explains why red pine suffers little inbreeding depression upon selfing, in marked contrast to other pines (e.g., Fowler, 1965). During the last glaciation, many forest trees were restricted to southern refugia, and one hypothesis has it that red pine lost its genetic variability as it persisted in a southern refugia (Fowler and Morris, 1977). During the Wisconsinian, glaciers covered nearly all of the present range of red pine, and indeed period-spanning macrofossils have been found in northern Georgia (Jackson et al., 1997, 2000; S. T. Jackson, personal communication), indicating that red pine did persist in a southern refugium. However, the loss of genetic variation, or at least lack of genetic variation, may alternatively extend much further back in evolutionary history. Red pine is evolutionarily distinct from all other North American pines except for *Pinus tropicalis*, a Caribbean endemic, and together these two are the only representatives of the subsection *Sylvestres*, which contains many Eurasian species. An estimate, based on molecular data, of the age of the split between the red pine lineage and that containing all other North American hard pines apart from *P. tropicalis*, is 120 million years ago (MYA) (Krupkin et al., 1996). Moreover, several late Cretaceous fossil species, such as *P. clementsii*, in North America bear remarkable resemblance to *P. resinosa*. *Pinus resinosa* may have existed as a distinct lineage for many millions of years.

Among seven studied populations in the northern half of the range of red pine, one in New Brunswick had much greater diversity for cpSSR loci (Echt et al., 1998). Coincidentally, among 10 studied populations spread over the southern half of the range, haplotype diversity values for a somewhat different set of cpSSR loci were much larger for three populations in Maine (0.653), New Hampshire (0.277), and Vermont (0.254) than the mean value of 0.028 (Walter and Epperson, 2001). This suggested but did not prove that there may be an area centered in northern New England where populations have unusually high levels of diversity. This entire area was

buried by glaciers during Wisconsin glaciation, and hence cannot be the origin of post-glacial red pine. Such a center of diversity would constitute a contradiction to standard theory about the patterns that evolve when a species spreads from a single origin. If a system has not reached equilibrium, then genetic diversity is greatest at the origin; if it has, then diversity levels should be uniform throughout the system. The situation in red pine appeared to be rather more complex.

Although there may be other factors, the possible role of other refugia, in addition to the one known in the southern Appalachians, would be critical. Even a small second refugial population in the north could have great consequences for the present pattern of genetic variation if it greatly expanded in size as new habitat opened. During the glacial maximum, a series of large islands and extensions of the mainland were exposed due to the reduced level ocean. Some parts of Newfoundland, Gulf of St. Lawrence, and Nova Scotia, as well as then-exposed land that is presently below sea level, was free of all glaciers (e.g., Grant, 1977; Ives, 1978; Brookes, 1982; Josenhans and Lehman, 1999). Studies of fossil flora from that region and time have rarely if ever been done, but the possibility that a red pine refugium existed there should not be discounted. Moreover, biologists have examined data on the present-day biotic distribution, and have widely accepted that there were well-developed communities in nonglaciated areas of the exposed continental shelf. For example, there are high concentrations of endemic races and subspecies for plants, insects and other animals in Nova Scotia and surrounding areas (e.g., Hamilton and Langor, 1987; see also review in Pielou, 1992). Moreover, it appears that red pine reached the previously glaciated New England remarkably early (e.g., Davis, 1983; Delcourt and Delcourt, 1981; Watts, 1983), although researchers rarely distinguish fossil pollen of *P. banksiana* from *P. resinosa*.

Evidence of a northern red pine refugium could be gained by examining the geographic distribution of genetic variation in populations in New England and surrounding areas. The present day geographic distributions of haplotypes is related to the histories of distinct lineages, because the chloroplast genome does not recombine (e.g., Neale and Sederoff, 1989). If the postglacial history of red pine is based on a single south-

TABLE 1. Sampled populations, origin named by state or province and county, codes,^a and latitude and longitude (in decimal degrees).

Provenance	Code	Latitude/longitude
<i>Previous study</i>		
Wisconsin/St Croix	776	45.25/92.03
Maine/Washington	758	45.00/67.75
New Hampshire/Merrimack	759	43.17/71.78
Vermont/Chittenden	739	44.63/73.13
New York/Franklin	705	44.48/74.28
Pennsylvania/Wyoming	742	41.52/76.15
Pennsylvania/Clinton	732	41.27/77.80
West Virginia/Hardy	WVH	39.00/78.75
West Virginia/Pendleton	WVP	38.75/79.25
Illinois/LaSalle	ILL	41.53/88.70
<i>Added in this study</i>		
Maine/Buckfield	745	44.33/70.25
New Brunswick/Queen's	723	45.75/66.33
New Brunswick/Kent	725	46.63/65.55
New Brunswick/Northumberland	727	47.18/65.43
New Brunswick/Victoria	768	47.00/67.40
Nova Scotia/Hants	791	45.15/63.97
Nova Scotia/Queens	793	44.33/65.15
New Hampshire/Hillsboro	760	43.12/71.67
New York/Warren	743	43.57/73.82
Quebec/Quebec	728	46.87/71.38
Quebec/Berthier	747	46.38/73.32
Ontario/Renfrew	746	45.68/77.08
Ontario/Nipissing	787	45.83/78.00
Ontario/Sudbury	788	46.58/82.25
Ontario/Algoma	789	46.75/83.92
Ontario/Kenora	790	49.75/93.12
Michigan/Wexford	757	44.25/85.50
Wisconsin/Oneida	771	45.60/89.67
Wisconsin/Eau Claire	772	44.78/91.37

^a Numeric codes are accession numbers used by the MICHOTIP collections of the Department of Forestry of Michigan State University; the lettered codes are populations sampled directly.

ern refugium model, then it seems unlikely that the species gained variation as it spread north or lost variation in the south (due to fragmentation), and then lost the same variants as it spread westward. A more parsimonious explanation is that a second, northern refugium existed. As lineages expanded their ranges from both refugia, they would have formed a contact zone. This contact zone would have greater diversity if the two refugia were genetically differentiated. Thus, a viable hypothesis is that the New England area is an admixture zone. In this study, we examine cpSSR loci for a large number of populations in and around New England in order to determine if indeed there is a center of diversity, and if it has characteristics of an admixture zone. Moreover, we address questions about how gene flow and selection might influence the shape of the zone, as well as the nature of geographic transitions from areas of high diversity to areas with very low diversity.

MATERIALS AND METHODS

Sampling—Needle tissues were sampled from a total of 313 individuals. Nineteen populations were chosen spanning a wide east/west range from Nova Scotia (longitude 63.97) to western Ontario (93.12), from those available in holdings in Michigan State University's MICHOTIP provenance (common garden) plantations (Table 1). For most populations 15–20 trees were sampled, but for two populations in Nova Scotia, one in New Brunswick, and one in Ontario fewer individuals (13, 5, 7, and 11, respectively) were available.

DNA isolation—Needle tissue (~100 mg) was cut into small pieces with a razor blade and placed in a 2 mL screw cap centrifuge tube (Molecular Express Ames, Iowa, USA) between a ceramic ball and a ceramic cylinder (both BIO 101 Savant Instruments, Holbrook, New York, USA). A 750- μ L cetyltrimethylammonium bromide (CTAB) solution was added, and the samples were ground for 45 s at the 4.5 setting in a FastPrep instrument BIO 101 Savant (BIO 101). After incubation at 65°C for 20 min (lysis), the homogenate was transferred into a centrifuge tube unit and DNA isolation was performed applying the automatic DNA isolation system "AutoGen 850" (AutoGen, Holliston, Massachusetts, USA), at the Genomics Technology Support Facility of Michigan State University. DNA concentrations were measured by fluorometry, and the yield was 10–80 μ g/extraction.

Chloroplast markers scored—Sampled trees were genotyped for the same 10 mononucleotide microsatellites reported in our previous study (Walter and Epperson, 2001). The primers were developed by Vendramin and colleagues (1996) based on known sequences in *Pinus thunbergii*. The designations of primer pairs, in terms of original (PT) numbers and the shorter nomenclature used for discussion here (in parenthesis), are: PT9383 (*cp2*); PT15169 (*cp3*); PT30204 (*cp5*); PT36480 (*cp6*); PT41093 (*cp7*); PT48210 (*cp9*); PT171936 (*cp12*); PT87268 (*cp14*); PT107148 (*cp17*); and PT110048 (*cp20*). The forward primer of each pair was labeled with one of 6- FAM, HEX, or TET (ABI) phosphoramidites. Overlapping allele size ranges were differentiated by using different dye labels in multiplexing.

PCR amplification and multiplexing—All polymerase chain reactions (PCRs) were carried out using 20 ng of template DNA in a total volume of 10 μ L. Optimized conditions for all reactions were achieved with a PCR buffer (20 mmol/L Tris-HCl [pH 8.75], 10 mmol/L KCl, 10 mmol/L (NH₄)₂ SO₄, 2 mmol/L Mg SO₄, 0.1% Triton X-100, 100 μ g/mL bovine serum albumin [BSA], 6% sucrose and 0.1 mmol/L cresol red), 3.5 mmol/L Mg Cl₂, 200 μ mol/L each dNTP, 250 μ mol/L each primer, and 0.25 units of AmpliTaq Gold (Perkin Elmer, Wellesley, Massachusetts, USA). The PCR amplifications were performed in an MJ Research (Waltham, Massachusetts, USA) thermocycler Model PTC-100, applying a touchdown amplification protocol: three step cycling with two cycles at 94°C for 1 min, 65°C for 1 min, 70°C for 35 s; 18 cycles at 93°C for 45 s, 64°C for 45 s (with lowering the annealing temperature from 64°C to 55.5°C by the 18th cycle), 70°C for 45 s; and 20 cycles at 92°C for 30 s, 55°C for 30 s, 70°C for 60 s followed by a final extension step at 70°C for 5 min.

Fluorescently labeled PCR products were multiplexed on a 0.40 mm, 6% denaturing polyacrylamide gel (7 mol/L urea) along with a 350-base pair (bp) size standard and an external standard (to control for any possible intergel size variation, which in fact did not occur). Samples were electrophoresed at 2500 V/30 W for 5.5 to 6 h. Labeled microsatellite fragments were run on a 373 ABI (Applied Biosystems, Foster City, California, USA) sequencer, using GeneScan, giving 1-bp resolution. As will be shown, a great majority of trees had the same haplotype, i.e., the same constellation of alleles at all loci. All sampled trees that had variant alleles were retested (re-amplified and genotyped) twice, and in all cases values were consistent. In addition, 256 or 8% of the total (313 trees \times 10 loci, or 3130) single-locus genotypic scores for common alleles were designated for random retesting, and again there were zero errors of genotyping.

Statistical analysis—Standard measures of genetic diversity and differentiation were calculated for haplotype frequencies using Lewis and Zaykin's (2001) GDA program (version 1.0 d16c). For each population sample, the number of haplotypes observed and effective "heterozygosity" or diversity of haplotypes (Weir, 1996) was calculated. Weir's "H" estimate of the average amount of differentiation of haplotype frequencies among populations was also estimated. Nei's (1978) measures of genetic distances among pairs of populations were used for the Mantel spatial statistics. Special diversity statistics designed to exploit differences in the numbers of repeats among alleles (e.g., "R_{ST}," Goldstein et al., 1995) were not appropriate for our data set. Polymorphic loci had only two or three alleles, and in the latter cases at least one allele was in very low frequency. Thus there cannot be substantial ad-

ditional information to exploit, and the additional assumptions, which might be violated, need not be made.

The genetic distance matrix could not be used to construct Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrograms, because of various issues, including occurrences of many "ties," where multiple pairs had the same distance values. It is worth noting that loci are not independent, because of the lack of recombination. Loci are completely dependent with respect to dispersal and drift, but not for mutation. Moreover, in our data, essentially all of the information on locus-specific alleles is contained in the haplotypes themselves. In effect, the treatment of haplotypes themselves as "alleles" of a locus is for our purposes consistent with infinite "alleles" (haplotypes) assumptions. Under the infinite alleles mutation model with genetic drift, Nei's measure is linear with time (Nei, 1987), if there is no migration (although it should be noted that the assumption of no migration is invalid in this as well as most other studies of genetic variation within species).

All spatial statistics were calculated using the PASSAGE program of Rosenberg (2002). Moran's I statistics were calculated for each mean-adjusted variable Z_i for population sample i , by $I = (\sum_i \sum_j w_{ij} Z_i Z_j) / (W \sum_i Z_i^2)$, where n is the number of populations, w_{ij} is a measure of weight reflecting spatial proximity for a pair of populations i and j , and W is the sum of all weights (Cliff and Ord, 1981). In the first instance, the w_{ij} are binary indicators of inclusion (1) or exclusion (0) of a pair of populations in a particular distance class, or range of geographic distances, using great circle distances separating populations. A set (correlogram) of the unweighted statistics for mutually exclusive and exhaustive distance classes was calculated. To determine if there is finer scale autocorrelation of genetic diversity within distance class one, we calculated weighted I statistics, such that pairs of populations within distance class one are weighted by inverse functions of distance of separation. Spatial directionalities were determined by calculating circular spatial autocorrelation statistics (Oden and Sokal, 1986), where pairs of populations are classified by distance and compass directions between populations. In all of the above cases, the expected value of I statistics under the null hypothesis of a spatially random distribution is equal to $-1/(n-1)$, or -0.036 . Standard errors and tests of significance were based on the randomization null hypothesis. Spatial statistics could be utilized for only some of the haplotypes, not for those (haplotypes V, VII, VIII, and IX) that occurred in only one population. The geographic distribution of haplotype diversity values was analyzed using the same spatial statistical methods. In addition, Mantel statistics were calculated to measure the overall association of genetic distance with geographic distance (Cliff and Ord, 1981). In this case, the small Illinois sample (the entire population consisted of four trees, all with haplotype I, and it is located hundreds of kilometers from any other natural stands of red pine) was excluded as potentially having over-influence. The two West Virginia samples were combined in order to have a better representation of the most south-eastern region, and for similar reasons the two Pennsylvania population samples were combined.

RESULTS

Six of the cpSSR loci, *cp2*, *cp5*, *cp6*, *cp7*, *cp9*, and *cp17*, were monomorphic. Two of the polymorphic SSRs, *cp3* and *cp12*, had two alleles, and two, *cp14* and *cp20*, had three alleles (Table 2). In total, there was an average of 1.6 alleles per locus and 2.5 per polymorphic locus. Nine haplotypes (Table 2) were observed among the 449 trees. Haplotype I was by far the most common and had a frequency of 0.869 in the entire data set (Table 3). It is widespread and found in nearly every population sample. Haplotype II was the next most frequent, and it had a much lower total frequency of 0.060. Four haplotypes, V, VII, VIII, and IX, were each found in only a single tree; haplotypes III and VI had relatively high frequencies of 0.018 and 0.038, respectively; and the remaining haplotype, IV, occurred in three trees (0.007). All haplotypes other

TABLE 2. Definition of haplotypes in terms of fragment sizes for polymorphic cpSSRs.

Haplotype	cpSSR locus and PT no. ^a			
	cp3	cp12	cp14	cp20
	15169	71936	87268	110048
I	123	149	164	95
II	123	148*	164	95
III	123	149	164	96*
IV	123	149	164	94*
V	123	149	162**	95
VI	123	149	163*	95
VII	124*	149	164	95
VIII	123	149	163*	94*
IX	123	148*	164	96*

^a *Pinus thunbergii* (PT) numbers are as defined by Vendramin et al. (1996). An asterisk denotes a single base-pair difference from haplotype I; two asterisks denote a two base-pair difference.

than I and II exhibit some degree of restricted range (Table 3).

Most of the phylogenetic relationships among haplotypes were clear from inspection; however, these were verified by cladistic analysis using PAUP branch and bound (Swofford, 2002), with four polymorphic loci (characters), each with ordered states. Haplotypes II, III, IV, VI, and VII all differ from haplotype I by a single base pair change in fragment size at one of the four polymorphic SSRs. In all most parsimonious cladograms, haplotype I is the most interior node, and haplotypes II, III, IV, VI, and VII are all one step to the exterior (Fig. 2). Haplotype V differs from I solely in that its fragment size (162) for cp14 is 2 bp smaller than that (164) for I, and differs from VI solely in that VI has the intermediate size (163); thus V appears to have arisen from VI. Mutational paths to haplotypes VIII and IX, both differing from I by a single base-pair difference at each of two SSRs, cannot be resolved by parsimony alone. For haplotype VIII, VII and IV are equally parsimonious intermediates. Similar roles are represented by II and III in the derivation of IX. However, based on the geographic distribution of haplotypes, VIII is more likely derivative of VI than of IV. The one tree that carried haplotype VIII was found in the same New Hampshire sample in which seven other trees with haplotype VI were found. All of the other 10 trees with haplotype VI were found in one of the isolated populations in West Virginia. In contrast, haplotype IV was found in three trees in total, and two of these were in Ontario, although one was found in a different New Hampshire sample. Similarly, haplotype IX appears somewhat more likely derived from II than from III. Haplotype II is more frequent than III (27 compared to 8 trees in total) and it is more widespread. In the eastern Ontario population (787) in which the only tree with haplotype IX was found, all of the other trees had haplotype I. However, haplotype II was found in the spatially proximal population 746. In contrast, haplotype III was found only in the northeast extreme of the species range (New Hampshire, Maine, and Nova Scotia). The diversity of haplotypes, H_e , within populations averaged 0.152, but exhibited a wide range. Many population samples had zero diversity, but values ranged up to 0.653, more than four times the mean. The number of haplotypes averaged 1.86, with a range from one to four. The only private haplotypes, i.e., restricted to a single population, were the four that each were found in a single tree, as mentioned above. All populations with high diversity are concentrated into a narrow, band-

TABLE 3. Incidences and diversities of haplotypes within study populations.

Population	n	Numbers of each haplotype									A*	H _e #
		I	II	III	IV	V	VI	VII	VIII	IX		
<i>Previous study</i>												
776 (WI)	20	19	0	0	0	1	0	0	0	0	2	0.097
758 (ME)	16	7	3	6	0	0	0	0	0	0	3	0.653
759 (NH)	20	17	1	1	1	0	0	0	0	0	4	0.277
739 (VT)	14	12	2	0	0	0	0	0	0	0	2	0.254
705 (NY)	20	20	0	0	0	0	0	0	0	0	1	0
742 (PA)	4	0	4	0	0	0	0	0	0	0	1	0
732 (PA)	20	19	1	0	0	0	0	0	0	0	2	0.097
WVH (WV)	10	0	0	0	0	0	10	0	0	0	1	0
WVP (WV)	8	8	0	0	0	0	0	0	0	0	1	0
ILL (IL)	4	4	0	0	0	0	0	0	0	0	1	0
<i>Added in this study</i>												
745 (ME)	15	12	3	0	0	0	0	0	0	0	2	0.331
723 (NB)	20	18	2	0	0	0	0	0	0	0	2	0.180
725 (NB)	20	20	0	0	0	0	0	0	0	0	1	0
727 (NB)	15	15	0	0	0	0	0	0	0	0	1	0
768 (NB)	7	7	0	0	0	0	0	0	0	0	1	0
791 (NS)	13	10	1	1	1	0	0	0	0	0	4	0.406
793 (NS)	5	4	1	0	0	0	0	0	0	0	2	0.356
760 (NH)	18	10	0	0	0	0	7	0	1	0	3	0.552
743 (NY)	15	11	4	0	0	0	0	0	0	0	2	0.404
728 (QU)	20	20	0	0	0	0	0	0	0	0	1	0
747 (QU)	20	19	1	0	0	0	0	0	0	0	2	0.097
746 (ON)	20	19	1	0	0	0	0	0	0	0	2	0.097
787 (ON)	20	19	0	0	0	0	0	0	0	1	2	0.097
788 (ON)	21	20	1	0	0	0	0	0	0	0	2	0.093
789 (ON)	11	11	0	0	0	0	0	0	0	0	1	0
790 (ON)	18	16	1	0	1	0	0	0	0	0	3	0.210
757 (MI)	18	17	1	0	0	0	0	0	0	0	2	0.108
771 (WI)	19	18	0	0	0	0	0	1	0	0	2	0.102
772 (WI)	18	18	0	0	0	0	0	0	0	0	1	0
Total/mean	449	390	27	8	3	1	17	1	1	1	1.86	0.152

* Number of different haplotypes.

Diversity of haplotypes.

shaped area centered in northeast New England. The overall measure of haplotype differentiation among populations, θ , for all haplotypes combined was 0.33 (Table 4), and this value is largely determined by frequency differences in haplotype I. Among separate measures of θ for each haplotype, values for the four unique haplotypes were near zero, but this is expected because θ is not well-defined in such cases. Among the other haplotypes, θ ranged from 0.18 to 0.73. The large value for haplotype VI (0.73) is striking and consistent with the fact that VI is found in only two samples, fixed in the sample from one of the isolated populations in West Virginia, and in high frequency in one of the samples from New Hampshire. Values for haplotypes II and III were both similar to that for I, despite the fact that haplotype II is widespread like haplotype I, but haplotype III is found only in parts of New England.

In the spatial analyses using correlograms of Moran's I statistics, 10 mutually exclusive distance classes were used with the following upper bounds in kilometers: 259, 408, 539, 678, 803, 973, 1180, 1405, 1650, and 2239. Each distance class contained either 40 or 41 pairs of populations. For haplotype diversities (Fig. 3), the value for distance class one is very large, positive (0.31) and highly significant ($P < 0.01$), the value for distance two was fairly large (0.12) but not statistically significant, and values for larger distance classes were small and often negative. The correlogram shows that populations with high diversity are highly autocorrelated and concentrated into an area a few to several hundred kilometers

across. Most of the autocorrelation occurs within 259 km. The value (0.65) for distance class one when pairs are weighted by inverse distance is greatly increased above the unweighted value and is again significant at the 1% level (Table 4). This indicates that populations with high diversity are highly autocorrelated within distance class one, i.e., at an even finer scale, confined to a distance of about 100 km. Consistent, the value observed for weights by the inverse of distance squared is also very large, although not statistically significant.

Despite the fact that diversity levels per se are highly autocorrelated, the frequencies of the five nonprivate haplotypes are not. Out of 50 unweighted I statistics (five haplotypes times 10 distance classes), only two were nominally significant. For haplotype IV, there was a large negative value (-0.28) at distance class nine (1405-1650 km), and this reflects its restriction to the east (although it is found in both the northeast and southeast). Interestingly, the other significant value was a positive one (0.12) for haplotype III at distance class two (259-408 km), despite the fact that a large, although not significant, negative autocorrelation (-0.15) was observed for distance class one. This is consistent with the fact that haplotype III was found only in samples from Maine, New Hampshire, and Nova Scotia, but it also suggests that over this area its frequencies are highly variable. Values for distance class one are reported in Table 4, as are weighted values. Most importantly, the values do not indicate substantial autocorrelation in the frequencies of haplotype I. In sum, these results

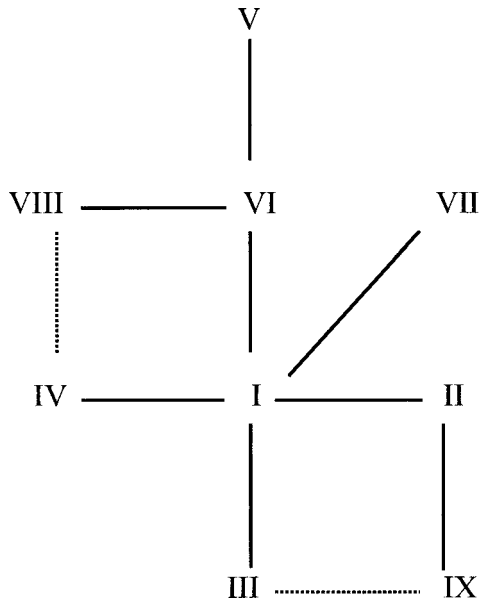


Fig. 2. Diagram of results of parsimony analysis. In all most parsimonious cladograms haplotype I is the most interior node. Haplotypes II, III, IV, VI, and VII all differ from I by a single base-pair change in fragment size at one of the four polymorphic SSRs. Haplotype V involves a two base-pair change from I at cp14, and a one base-pair difference from haplotype VI at the same locus, and thus is inferred to be derived from VI. There are two cases where haplotypes exhibit ambiguity with respect to parsimony. Haplotype VIII is a single change in state from both IV and VI and more precisely differs from each by a single one base-pair difference at one SSR. Analogous is the derivative nature of IX from II and III. Solid lines between II–IX and VI–VIII represent the more likely paths, and dotted lines between III–IX and IV–VIII represent the less likely paths, based on the geographic and frequency distributions.

indicate that although there is nonrandomness to the pattern of haplotypes and especially haplotype diversities, the observed spatial pattern is not consistent with equilibrium isolation by distance models.

The Mantel test, measuring the overall association of genetic distance of haplotype frequencies among populations with geographic distances separating populations, was not significant. There is little genetic isolation by distance of the form generated by genetic drift and limited migration. However, the correlogram of Mantel values indicates some spatial structure

TABLE 4. Measures of spatial correlations and differentiation.

Haplotype	θ	Moran's <i>I</i> for distance class one†		
		un	wd	wds
I	0.33	-0.06	-0.10	-0.10
II	0.18	0.00	0.03	0.09
III	0.24	-0.15	-0.10	-0.10
IV	-0.01	-0.12	-0.24	-0.58
V	-0.02	—	—	—
VI	0.73	-0.03	-0.16	-0.38
VII	-0.01	—	—	—
VIII	-0.02	—	—	—
IX	-0.01	—	—	—
Combined	0.33	-0.07	-0.11	-0.23
H	—	0.31*	0.65*	1.29

† un, unweighted; wd, weighted by inverse distance; wds, weighted by inverse distance squared.

* Statistical significance at the one percent level.

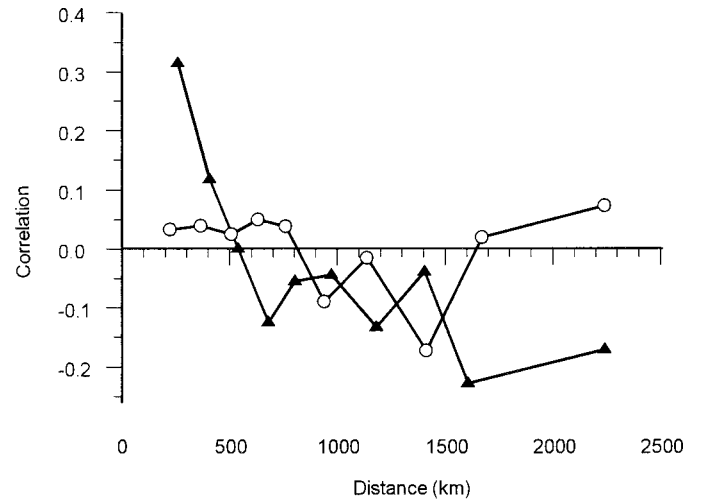


Fig. 3. Moran's *I* statistics for autocorrelation of genetic diversity, given by closed triangles, and Mantel statistics for genetic distances, given by open circles.

(Fig. 3). Most notably, there is a large negative and nominally significant ($P < 0.01$) value for distance class eight (1138–1412 km).

Directional autocorrelation analyses did not detect trends of haplotype frequencies with latitude or longitude or any other direction. Out of 40 statistics (eight distance/direction classes and five nonprivate haplotypes), only one was nominally significant and none of the five distance/direction correlograms were significant. In contrast, the distance/direction correlogram for diversity values was statistically significant ($P < 0.05$). The highest correlation was 0.38 ($P = 0.001$), and it occurred for populations separated by distances from 150 to 600 km (insufficient numbers of populations were separated by less than 150 km given the direction classes, hence directional autocorrelation cannot be calculated for distances less than 150 km) and bearing directions 0° to 60° , or a typical bearing of 30° . This indicates a high level of correlation at short distances along an essentially southwest to northeast axis. This is consistent with the map showing a tilted, band-shaped area of concentration of populations with high diversity along such an axis, in the northeast parts of New England extending northeast into Nova Scotia and the southeastern parts of New Brunswick (Fig. 4).

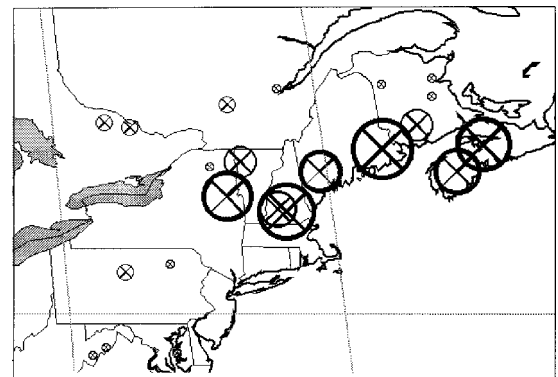


Fig. 4. Distribution of haplotype diversity values in the eastern part of the range. The diameters of the symbols are graduated according to inverse ranks, and the smallest shown had zero diversity.

DISCUSSION

Pinus resinosa has remarkably little variation for molecular genetic markers. For nearly 450 trees from 29 widespread populations, only four of 10 assayed chloroplast microsatellites were polymorphic, having either two or three alleles. Very similar results were obtained in previous studies of red pine (Echt et al., 1998; Walter and Epperson, 2001). In contrast, much larger numbers of alleles are typically found for cpSSRs in comparable studies of other pine species (Powell et al., 1995; Provan et al., 1999). A total of nine haplotypes were found.

The observed genetic differentiation among populations and the spatial pattern of genetic variation both indicate that there is little genetic isolation by distance over the species range. A low level of isolation by distance is broadly consistent with observations in other pines and with the high levels of dispersal of pollen and seed for red pine and typical of pines (Epperson, 2003). Moran's *I* statistics and Mantel statistics both showed that there is little spatial autocorrelation of the observed genetic variation in terms of haplotype frequencies. Haplotype I had by far the greatest total frequency (0.869), and it is very widespread, indeed found in nearly every population. The second most frequent haplotype, II (0.060), is also widespread. Four haplotypes, V, VII, VIII, and IX, were each found in only a single tree, each in quite different regions. While it is possible that each one of these occurs in regions outside where it was found, albeit at low frequencies and not sampled, another explanation is that each is a fairly recent mutation. All four are at exterior nodes of the cladogram (Fig. 2). The remaining haplotypes, III, IV, and VI, all have low global frequencies (0.007–0.038) and show various degrees of geographic restriction.

Despite the low amount of genetic isolation by distance, genetic variation exhibits a strikingly nonrandom spatial pattern that indicates limitations to gene flow are controlled by latitude rather than distance. Haplotype diversity values are highly autocorrelated. The Moran statistic for distance class one (259 km) was very large (0.31) and significant, the value for distance two was considerably smaller (0.12), and values for larger distance classes were small and often negative. This is due to a strong concentration of populations with high diversities in parts of New England and immediate surroundings. However, even within this area high diversity values are further concentrated, as indicated by analyses of weighted Moran *I* statistics within distance class one. Populations with the highest diversity are concentrated into a narrow band (Fig. 4), and the orientation of this is on a southwest to northeast axis, as was determined by directional autocorrelation analysis. The results indicate that there is little gene flow north or south across this band. It is worth pointing out that this area is often not represented in red pine experiments on genetics of morphological traits and provenance trials.

The results also reveal remarkable narrowness of the area and sharpness of its boundaries. The boundaries have not been smoothed as would be expected from even fairly low levels of gene flow. All of the sampled populations in eastern Ontario and southern Quebec have low diversity, as do the even more spatially proximal areas of northern New Brunswick and northern New York. In contrast, all of the populations in the band-shaped area have high levels of diversity, including the one (743) more southeast in New York. In summary, all and only populations with high diversity occur in a narrow band

extending from southeastern New York toward the northeast through New Hampshire and Maine, to Nova Scotia and southern New Brunswick. This is wholly inconsistent with equilibrium models of selectively neutral markers under isolation by distance (e.g., Epperson, 2003), which would predict that diversity level is uniform over the species range. Red pine is either not as near equilibrium as would be expected based on its high levels of dispersal of seed and pollen, or the cpSSRs have been subject to selection (if so, most likely as hitchhiking effects), or both. Selection could cause such a pattern either at equilibrium or with nonequilibrium and cannot be excluded. Nonetheless, it is also likely that the distribution is not near equilibrium despite the fact that high levels of gene flow are expected based on the dispersal characteristics of seed and pollen.

Because the geographic pattern of haplotypes is entirely inconsistent with equilibrium neutral models, explanations should focus on processes that are not at equilibrium. One of the simplest is that the area with relatively high diversity was the origin of red pine as the glacier retreated, and genetic diversity was lost in populations expanding from it. However, this area was buried by the glaciers and thus cannot be the origin. Moreover, it is known that red pine did persist in the south, because macrofossils of red pine, dating from 16 000 and 18 000 yr ago, have been found in northern Georgia. Generally, eastern pines are considered to have survived in the southeastern United States and expanded northward after the glacial maximum. Among the newly sampled populations, it is most telling that haplotype VI was found only in a New Hampshire population, whereas previously it was found only in one of the populations in West Virginia: indeed in that sample all 10 trees were haplotype VI. This strongly suggests that the populations in the high diversity zone have been influenced by the southern refugia.

It appears unlikely that a single southern refugium model could produce the geographic pattern. First, it would require either that red pine gained variation as it moved northward but not in the south or it lost variation in the south after it expanded northward. The latter is reasonable, since decreases in regional population size and fragmentation and isolation clearly have occurred in the southeast. The southeastern populations sampled do have low diversity and relatively high differentiation. Most striking is the fixation of different haplotypes in the samples from the two West Virginia populations that are separated by only 50 km. Second, red pine would have to have lost genetic variation again as it spread from New England westward to its range boundaries. It would have had to lose precisely the same variants it had either gained in the northeast or lost in the southeast during the first phase, which seems unlikely. When red pine spread toward the west it did so as a rapidly expanding regional population (Davis, 1983), which remains very large today. Throughout the modern northern range of red pine there are millions of red pine trees. Substantial drift and particularly loss of variants are unlikely for a large and rapidly growing regional population.

In contrast, the observed geographic pattern is entirely consistent with there having been two refugia, one in the northeast in addition to that in the southern Appalachians. Thus, while it may be possible to construct other more complicated nonequilibrium processes that are consistent with the data or to invoke various selection models, we focus on this possibility as more parsimonious. Geologists have increasingly emphasized that there were unglaciated areas in the northeast (Grant,

1977; Ives, 1978; Brookes, 1982). Many ecologists have long been convinced that terrestrial ecosystems persisted there as refugia throughout the Wisconsinian (e.g., Fernald, 1911; Pielou, 1992). During the glaciation, the level of ocean dropped, exposing a series of islands and extensions of the mainland from Newfoundland extending well south of Nova Scotia. Much of this exposed area (as well as small parts of the present coast line) was not covered by the Laurentide ice sheet, and much of it also was not covered by smaller glaciers. Unfortunately, there has been very little study of fossil flora in this region, much of which is now covered by the Atlantic, and we know of no finds of red pine fossils in this region. However, it is known that period mastodon fossil bones are frequently found by fisherman trawling in areas that were above the Wisconsinian sea level and that mastodons primarily consumed conifers, including pines (Pielou, 1992). The two refugial populations could have expanded and met in the area where 10-fold greater diversity is found today. It is possible that much of the discrepant paucity of genetic variation in red pine relative to other pines could have been in place well before the Wisconsinian. If so, then there would have been only minor genetic differentiation between the two refugia, caused both by decreasing population sizes as they contracted into the refugia and by mutations that occurred during isolation. Nonetheless, with respect to the genetic diversity of the entire species today, that differentiation would be the major controlling factor. Very recently, other researchers have developed several polymorphic nuclear SSRs in *Pinus resinosa*, and they found that populations in Newfoundland and certain parts of New Brunswick were highly differentiated from other populations, strongly supporting our interpretation that there was a second, northeast refugium (J. Boys, M. Cherry and S. Dayanandan, unpublished manuscript). Moreover, they found that the diversity of nSSRs was markedly higher in the same general region as the band-shaped area of high cpSSR diversity, supporting the idea that this area is a contact zone.

It appears that there were two refugia, and if both had significant influences on red pine today, then haplotype I was common in both. The fact that haplotype I has very high frequency throughout the range somewhat limits distinctions that can be made between two refugial lineages. Haplotype II is both the second most frequent and is also widespread, being repeatedly found in all regions, the northeast, south, and northwest. If there were two important refugia, haplotype II was probably present in both. In contrast, the distribution of haplotype VI suggests that it was only in the southern refugia. Haplotype VIII is most likely a recent mutant of haplotype VI, and it was found in a single tree, in the same population in New Hampshire. Other haplotypes, V, VII, and IX, were also found in single trees and likely are recent mutants. It is worth noting that haplotype V is very likely (barring a 2-bp mutation) a mutant of VI and was found only in a single tree in central Wisconsin, near the southern limit of the present western range. This suggests that the southern refugia did spread far west. Haplotype VII is almost certainly a mutant from haplotype I and hence could be derived from either refugium. Haplotype IX is more likely a mutant of II and also could be from either refugia. Most informative is that haplotype III is found only in the area of high diversity. It is far more common near the eastern end of the band, in Nova Scotia and eastern Maine, and outside of this more restricted area it was found only in a single tree in New Hampshire. Thus haplotype III would likely be from the northeastern refugium. Haplotype IV

is quite interesting in that it was found in the far northwest edge of the species range, and at similar latitudes in New Hampshire and Nova Scotia, suggesting it was derived from the northern refugium. Overall, the results suggest that if a second refugium did exist in the north and then spread, it did so along a northern latitudinal band. The southern refugium would have spread somewhat north and then west along a southern latitudinal band. This would further suggest that the two may differ in their fitness with respect to latitude, as might be expected based on adaptiveness of photoperiodic responses.

In summary, a remarkably narrow and sharply bounded band-shaped area of high diversity was found. One might have expected to find intermediate diversity in nearby regions, for example in southeastern Quebec. Instead there is a sharp drop. Further, northern New Brunswick and similar latitudes in Quebec and eastern Ontario are nearly fixed for haplotype I. If the band of high diversity is an admixture zone, it appears to have remained remarkably tight since forming some 10 000 yr ago, when the pollen record indicates red pine had reached this region (Davis, 1983). Why would not pollen and seed dispersal have mixed the lineages and smoothed out the admixture area? One answer, consistent with the observed patterns for both haplotype frequencies and genetic variation of photoperiodic and other morphological traits, is that two refugial lineages have spread essentially westward along latitudinal lines, because of latitudinal selection between the lineages. Recent studies that have detected marked genetic differentiation associated with selection mediated environmental factors include Mitton and Duran (2004). It should be noted that a potential alternative is that the contact zone is very recent.

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