Evidence from sequence-tagged-site markers of a recent progenitor-derivative species pair in conifers

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Black spruce (*Picea mariana* [B.S.P.] Mill.) and red spruce (*Picea rubens* Sarg.) are two conifer species known to hybridize naturally in northeastern North America. We hypothesized that there is a progenitor-derivative relationship between these two taxa and conducted a genetic investigation by using sequence-tagged-site markers of expressed genes. Based on the 26 sequence-tagged-site loci assayed in this study, the unbiased genetic identity between the two taxa was quite high with a value of 0.920. The mean number of polymorphic loci, the mean number of alleles per polymorphic locus, and the average observed heterozygosity were lower in red spruce ($P = 35\%$, $A_P = 2.1$, $H_o = 0.069$) than in black spruce ($P = 54\%$, $A_P = 2.9$, $H_o = 0.103$). No unique alleles were found in red spruce, and the observed patterns of allele distribution indicated that the genetic diversity of red spruce was essentially a subset of that found in black spruce. When considered in combination with ecological evidence and simulation results, these observations clearly support the existence of a progenitor-derivative relationship and suggest that the reduced level of genetic diversity in red spruce may result from allopatric speciation through glaciation-induced isolation of a preexisting black spruce population during the Pleistocene era. Our observations signal a need for a thorough reexamination of several conifer species complexes in which natural hybridization is known to occur.

allopatric speciation | genetic diversity | natural introgressive hybridization | Pleistocene | red spruce

Genetic evidence in support of progenitor-derivative species relationships in plants was first reported within *Stephanomeria* and *Clarkia* (1, 2). Several progenitor-derivative species pairs have since been identified in other annual plant taxa (ref. 3 and reviewed in ref. 4), and cases have also been reported in several perennial plant taxa (e.g. refs. 5–8). Genetic characteristics considered indicative of a progenitor-derivative relationship include: (*i*) a high degree of genetic similarity between species, (*ii*) a low level of genetic diversity in the derivative species, (*iii*) fewer alleles in the derivative species, and (*iv*) few unique alleles in the derivative species, in comparison to the progenitor (1, 9). Indeed, the genetic diversity of a derivative species is generally a subset of the progenitor's gene pool. A derivative species usually has a narrower natural geographical range and is more ecologically restricted than is its progenitor (e.g., refs. 1, 4, and 6), and, frequently, it can interbreed with the progenitor to form hybrids (e.g., refs. 4, 5, 7, and 10–12).

Coniferous species are among the most genetically diverse of organisms (13), although there are infrequent reports of species having drastically reduced genetic diversity (14–17). Natural interspecific hybridization between closely related conifer taxa has also been reported in several instances (18–24), but overall, no clear evidence that would substantiate the existence of any putative progenitor-derivative species pairs has been presented.

Direct molecular evidence of natural introgressive hybridization between the closely related red spruce (*Picea rubens* Sarg.) and black spruce (*Picea mariana* [B.S.P.] Mill.) in eastern Canada has recently been obtained by using species-specific genetic markers (25). Not only is natural introgressive hybridization extensive in a broad portion of the area of sympatry shared by the two taxa, but introgression, albeit at a low level, was also evident in allopatric populations (25). Red spruce has a small to medium natural geographical range restricted to northeastern North America (26). It also has a restricted ecological amplitude, being a minor component of mixed forests on cool and well drained microsites (27). On the other hand, black spruce is an abundant transcontinental boreal species in North America, where it is found in a variety of stand types, often as the dominant or even the only tree species (26, 28).

Based on allozymes, red spruce seems to be genetically depauperate in comparison to other boreal conifers (29, 30). Conversely, black spruce has been reported to possess high genetic diversity, based on allozymes and genotypic data at random amplified polymorphic DNA (RAPD) loci (31), similar to levels reported for the sympatric but phylogenetically more remote *Picea glauca* (32). In addition, based on a set of 158 putative RAPD loci and comparing polymorphism within and across species, allopatric red spruce was found to be much less diverse than allopatric black spruce, with the percentage of polymorphic fragments for the two species being 5.3% and 22.5%, respectively (33). Although biogeographical factors and possible bottlenecks related to the recent ice age have been invoked to account for the low genetic diversity of red spruce and its marginal ecological amplitude (29, 30), we further hypothesize that these effects might have resulted in the recent speciation of red spruce from a preexisting black spruce population.

The objective of this study was to seek evidence to support, at the genetic level, the hypothesis that there is a progenitor-derivative species relationship between black spruce and red spruce, the former being the hypothetical progenitor. Genetic diversity was evaluated by using sequence-tagged-site (STS) markers of expressed genes. These are Mendelian, codominant markers resulting from the amplification of well characterized specific loci (34), thus leading to a precise estimation of the genetic diversity within and between species.

Materials and Methods

Sampling and DNA Isolation. For each species, foliage was collected from 45 mature trees that were present in test plantations located near Quebec City, Canada, and representative of three allopatric populations of the Canadian boreal forest for black spruce, and

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Abbreviations: RAPD, random amplified polymorphic DNA; STS, sequence-tagged-site.

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Table 1. Genetic diversity in populations of black spruce and red spruce based on STS loci

Populations	N_S	N_R	26 STS loci				14 polymorphic STS loci			
			A	*P*	H_O	H_e	*A*	*P*	H_O	H_e
Black spruce										
Area of allopatry*	45	43	1.9	46	0.097 (0.033)	0.104 (0.035)	2.6	86	0.180 (0.053)	0.193 (0.054)
Area of sympatry†	100	39	1.8	50	0.109 (0.033)	0.137 (0.038)	2.6	93	0.202 (0.049)	0.254 (0.054)
Both areas	145	82	2.0	54	0.103 (0.032)	0.122 (0.036)	2.9	100	0.191 (0.050)	0.226 (0.053)
Red spruce										
Area of allopatry‡	45	38	1.3	23	0.056 (0.031)	0.053 (0.027)	1.6	43	0.104 (0.055)	0.099 (0.048)
Area of sympatry§	100	47	1.5	31	0.079 (0.037)	0.088 (0.036)	2.0	57	0.147 (0.063)	0.163 (0.062)
Both areas	145	85	1.6	35	0.069 (0.033)	0.077 (0.032)	2.1	64	0.127 (0.057)	0.142 (0.054)

N_S, number of trees sampled; N_R, number of trees retained after rejection of hybrids/introgressants; *A*, mean number of alleles per locus; *P*, percentage of polymorphic loci; H_O, average observed heterozygosity (standard errors in parentheses); H_e, average expected heterozygosity (standard errors in parentheses).
*Sampling sites 1, 2, and 3, respectively, from Park Mistassini, QC (50.3°N 73.4°W), Manicouagan, QC (50.4°N 68.5°W), and Ipsala, ON (49.0°N 90.3°W).
†Sampling sites 4 and 5, respectively, from Villeroy, QC (46.2°N 71.5°W) and Tourville, QC (47.1°N 70.0°W).
‡Sampling sites 8, 9, and 10, respectively, from October M.S.F., MA (42.4°N 73.2°W), Glade Run, WV (38.6°N 79.8°W), and Indian Cap, NC (35.6°N 83.5°W).
§Sampling sites 6 and 7, respectively, from Duschesnay, QC (46.5°N 71.4°W) and Fredericton, NB (46.0°N 66.4°W).

three allopatric populations from the northeastern United States for red spruce (Table 1). In addition, 100 mature trees per species were randomly sampled from four stands in the area of sympatry in eastern Canada (Table 1). Stands were either dominated by black spruce (two) or red spruce (two). The exact geographical limit between the sympatric and the allopatric areas is difficult to define precisely and might rather be represented by a more or less diffuse zone, at least for red spruce in the northeastern United States. Hence, our allopatric and sympatric designations of populations followed previous characterization at the genetic level, which showed that the frequency of hybrids/introgressants is reduced by almost an order of magnitude in the sampled populations designated allopatric, as compared with the populations from the area of sympatry (25). DNA was extracted from needles by using the methods described in ref. 35 with minor modifications (33).

***A Priori* Detection and Exclusion of Hybrid/Introgressant Trees.** By using previously developed species-specific RAPD markers (33), the species identity of each sampled tree was assessed *a priori* to reject possible hybrid/introgressant trees. We followed this cautious approach to reduce biases in estimating genetic diversity for each taxon. One should note that relying solely on morphological characters to identify and exclude hybrids/introgressants is not a reliable procedure for this species complex (25). DNA amplifications were conducted following previously published procedures (33). Trees having combinations of species-specific RAPD markers from both taxa were classified as hybrids/introgressants and simply excluded from further analyses.

STS Markers. Primer pairs that direct the specific amplification of 26 STS loci of expressed genes were used (34). These markers were developed from arbitrary cDNAs that have been characterized at the sequence level; GenBank accession numbers, putative gene identifications, marker nomenclature, and primer sequences have been reported in detail (34). Indel polymorphisms (allelic length variants), detected among genomic amplification products by examination directly on agarose gels without further manipulation, have been characterized at the sequence level, and their Mendelian segregation has been assessed in heterozygous trees by using haploid megagametophytes (34). Most of these indel polymorphisms are found within introns or in the 3′ untranslated regions, and they vary in size from a few bp for some loci to a few hundred bp for others (34).

In this study, we used 12 loci previously shown to harbor exclusively codominant length polymorphisms in black spruce (*Sb01, Sb06, Sb07, Sb08, Sb11, Sb21, Sb24, Sb29, Sb31, Sb62, Sb70,* and *Sb72)* and a set of 14 loci (*Sb12, Sb14, Sb19, Sb28, Sb36, Sb41, Sb49, Sb50, Sb51, Sb56, Sb58, Sb60, Sb64,* and *Sb67*) chosen randomly among 22 candidate loci previously shown to be monomorphic in that species with regard to length variation (34). DNA amplification was conducted by using methods described in ref. 34 and by duplexing sets of primers that were predicted not to have serious primer interactions and generated products that differed sufficiently in size; 10 two-locus combinations of STS primers were used: (*i*) *Sb06-Sb70*, (*ii*) *Sb07-Sb72*, (*iii*) *Sb08-Sb41*, (*iv*) *Sb11-Sb49*, (*v*) *Sb12-Sb51*, (*vi*) *Sb14-Sb62*, (*vii*) *Sb19-Sb58*, (*viii*) *Sb28-Sb67*, (*ix*) *Sb29-Sb31*, and (*x*) *Sb60-Sb64*. The remaining loci (*Sb01*, *Sb21*, *Sb24*, *Sb36*, *Sb50*, and *Sb56*) were assayed individually. All primer pairs resulted in positive amplifications of red spruce DNA.

Tests were performed to verify whether allelic length variants were homologous between the two species with respect to indel structure. This procedure was accomplished by mixing templates of putative allelic homologues (including those fixed at monomorphic loci) from each species, followed by PCR and examination of products on agarose gels for the presence of more slowly migrating heteroduplex molecules. Tests were run in duplicate, and in no case was heteroduplex formation observed, indicating that the indel structure of presumed homologues was indeed the same.

Numerical Analysis. The proportion of polymorphic loci (*P*), the mean number of alleles per locus (*A*) and per polymorphic locus (A_p), allele frequencies at each locus, and observed and expected heterozygosities (H_o and H_e) were estimated for the 26 loci in each species and separately for trees from the areas of allopatry and for trees from the sympatric region. Heterogeneity of allele frequencies between black spruce and red spruce was tested by using *G* tests (35). Differences between species in allelic richness and heterozygosity were tested by using nonparametric Wilcoxon's signed-rank tests (36). Nei's unbiased genetic identities (*I*) and distances (*D*; ref. 37) were estimated within and between species.

Simulations. Simulations were conducted to test the hypothesis of a recent divergence of red spruce by genetic drift during the Pleistocene era (1.8 million years B.P. to 11,000 years B.P.), after a range of bottlenecks applied to a preexisting black spruce population, and assuming no migration nor mutation. The genetic diversity parameters monitored were the proportion of polymorphic loci, the average number of alleles per polymorphic locus, and the average expected heterozygosity at polymorphic loci. The initial population was composed of $2N_e$ gametes, where

Table 2. Allele frequencies and observed heterozygosities at 14 polymorphic STS loci* in black spruce and red spruce

		Allele frequencies						Observed heterozygoties			
		Black spruce		Red spruce		Heterogeneity *G* test†		Black spruce		Red spruce	
Locus	Alleles sizes, bp	Allopatric	Sympatric	Sympatric	Allopatric	Areas of allopatry	Areas of sympatry	Allopatric	Sympatric	Sympatric	Allopatric
Sb01	1,870	0.051	—	0.023	0.048	66.27‡	19.77‡	0.641	0.579	0.750	0.581
	1,900	0.077	0.171	0.125	—						
	1,930	0.538	0.592	0.432	0.306						
	1,960	0.077	0.105	0.364	0.629						
	2,010	0.167	0.079	0.034	—						
	2,075	0.064	0.053	0.023	0.016						
	2,125	0.013	—	—	—						
	2,155	0.013	—	—	—						
Sb06	539	0.940	0.974	1.0	1.0	6.46§	3.15	0.119	0.053	0	0
	609	0.060	0.026	—	—						
Sb07	645	—	0.066	—	—	—	6.97§	0	0.026	0	0
	648	1.0	0.934	1.0	1.0						
Sb08	634	0.038	0.041	—	—	30.57‡	9.78§	0.400	0.432	0.178	0
	645	0.175	0.230	0.322	—						
	646	0.738	0.581	0.622	1.0						
	653	0.050	0.149	0.056	—						
Sb11	691	0.122	0.145	0.044	—	13.48‡	5.14	0.195	0.132	0	0
	695	0.878	0.855	0.956	1.0						
Sb14	446	0.976	1.0	1.0	1.0	2.50	—	0	0	0	0
	500	0.024	—	—	—						
Sb21	471	0.131	0.224	0.033	0.068	1.78	15.55‡	0.167	0.289	0.067	0.027
	474	0.869	0.776	0.967	0.932						
Sb24	738	0.967	0.861	1.0	0.893	2.55	16.53‡	0.067	0.278	0	0.214
	771	0.033	0.139	—	0.107						
Sb29	553	0.155	0.158	0.022	—	18.89‡	14.04‡	0.286	0.316	0.044	0
	574	0.833	0.816	0.978	1.0						
	580	0.012	0.026	—	—						
Sb31	439	0.048	0.026	—	—	5.15	3.15	0.095	0.053	0	0
	449	0.952	0.974	1.0	1.0						
Sb51	345	—	0.013	—	—	—	1.57	0	0.026	0	0
	358	1.0	0.987	1.0	1.0						
Sb62	681	0.690	0.622	0.044	0.014	182.99‡	139.00‡	0.452	0.405	0.111	0.027
	689	0.143	0.054	0.011	—						
	691	0.036	0.054	0.944	0.986						
	706	0.131	0.270	—	—						
Sb70	404	0.024	0.066	—	—	199.50‡	109.83‡	0.048	0.184	0.400	0.054
	410	—	0.053	0.800	0.973						
	417	0.976	0.882	0.200	0.027						
Sb72	515	0.024	0.132	0.567	0.417	40.78‡	35.97‡	0.049	0.053	0.511	0.556
	523	0.976	0.868	0.433	0.583						
n of alleles		37	36	28	22						

*Other STS loci (12) tested were monomorphic in black spruce and red spruce (see *Results*).
†Heterogeneity *G* test (36) of allele frequencies between black spruce and red spruce.
‡Significant at $P < 0.01$.
§Significant at $P < 0.05$.

N_e is the effective population size, with allele frequencies corresponding to the current values observed in the overall black spruce population at 14 loci found to be polymorphic. Each new generation was a random sample of $2N_e$ gametes drawn from the previous generation. The population parameters tested were the effective population size N_e = 100, 1,000, 5,000, 10,000, 25,000, and 50,000 and the number of generations $t = 10^1$, 10^2, 10^3, 10^4, 10^5, and $10^{5.5}$, which spanned more than the entire length of both the Holocene (11,000 years B.P. to present) and Pleistocene eras assuming a generation time of 10 years. Pairwise combinations of parameters were each simulated independently with 10 replicates. The large N_e values tested are not unrealistic, based on effective population sizes estimated conservatively at many thousands of trees in black spruce and in excess of 10,000 for some other spruce species (38). Simulations were conducted on an Origin 2000 computer (SGI-CRAY, Mountain View, CA).

Results

***A Priori* Exclusion of Hybrid/Introgressant Trees.** The validation of sampled trees with species-specific RAPD markers resulted in the rejection of 10% of the trees from the areas of allopatry and 57% of the trees from the sympatric region (Table 1). This high rejection rate in the area of sympatry was consistent with reported results of extensive natural hybridization in that area (25).

Genetic Diversity at STS Loci. Of the 26 STS loci assayed, 14 loci were found to be polymorphic (Table 2), including 12 loci previously shown to harbor exclusively codominant length polymorphisms (34). For one of these, *Sb01*, three additional codominant rare alleles were detected. In the present study, new codominant length polymorphisms were detected at a low frequency for *Sb14* and *Sb51,* which were previously reported to be monomorphic (34). Of the 14 polymorphic loci, all were found to be polymorphic in black spruce, whereas nine were polymorphic in red spruce (Table 2), giving overall estimates of 54% and 35% of loci being polymorphic for each species, respectively (Table 1). If only the populations from the allopatric areas were considered, 46% and 23% of the loci were found polymorphic in black spruce and red spruce, respectively.

Heterozygosities were generally lower in red spruce than they were in black spruce. When all loci and individuals sampled from the sympatric region and the areas of allopatry were considered, the average observed heterozygosities were 0.069 in red spruce and 0.103 in black spruce (Table 1). When the areas of allopatry were considered alone, the respective observed heterozygosities were 0.056 and 0.097 (Table 1). In the sympatric region, red spruce had lower heterozygosity than black spruce at 10 loci; when individuals from allopatric areas were compared, this difference decreased to eight loci (Table 2). Both trends were significant at $P < 0.01$ (Wilcoxon's signed-rank test).

Overall, 41 alleles were detected in red spruce, giving an average of 1.6 alleles per locus, and 52 alleles were detected in black spruce, for an average of 2.0 alleles per locus (Table 1). This difference of 11 alleles was statistically significant ($P < 0.01$, Wilcoxon's signed-rank test). When only allopatric populations were considered, the respective numbers of alleles per locus were 1.3 and 1.9 (Table 1), and red spruce had a total of 15 alleles fewer than black spruce ($P < 0.01$, Wilcoxon's signed-rank test). No length-variant alleles were found to be unique to red spruce (Table 2).

At 10 of the 14 polymorphic loci in black spruce (*Sb06*, *Sb07*, *Sb08*, *Sb11*, *Sb14*, *Sb21*, *Sb24*, *Sb29*, *Sb31,* and *Sb51*), the most common alleles in black spruce were fixed or nearly fixed in red spruce (Table 2). This trend was most notable in populations from the areas of allopatry. At two other polymorphic loci (*Sb62* and *Sb70*), the common alleles differed between black spruce and red spruce. And, for the remaining two loci (*Sb01* and *Sb72*), the common alleles differed from black spruce in only part of the red spruce range. *G* tests of allele frequency heterogeneity between species were significant at $P < 0.01$ for many loci (Table 2).

The average Nei's genetic identity *I* between red spruce and black spruce was 0.920 ± 0.012 (average Nei's genetic distance $D = 0.083 \pm 0.013$), and pairwise *I* between populations of the two species ranged from 0.896 to 0.942. In the area of sympatry, the average *I* between species was 0.935 ± 0.006 ($D = 0.067 \pm 0.006$), and it was 0.920 ± 0.012 ($D = 0.093 \pm 0.008$) when only populations from the areas of allopatry were considered. Pairwise *I* values ranged from 0.978 to 1.0 between populations of red spruce and from 0.986 to 0.999 between populations of black spruce, with an overall mean of 0.992 ± 0.002 ($D = 0.008 \pm 0.002$) between populations within species. Thus, conspecific populations were nearly identical. No attempt was made to estimate the divergence time between the two species from their interspecific genetic distance because of the paucity of data on indel mutation rates at STS loci of plant nuclear genes.

Genetic Drift Simulations. With initial gamete frequencies corresponding to allele frequencies observed at the 14 polymorphic loci for the overall black spruce population, the three genetic diversity parameters monitored were quite synchronized in reaching the baseline level of genetic diversity observed in allopatric red spruce (Fig. 1). For an effective population size of 1,000, the baseline was reached after only 1,000 generations, which would point to the early Holocene era for the divergence of red spruce, assuming a generation time of 10 years. For N_e values of 5,000 and 10,000, the baseline was reached at around 10,000 generations, which would date the divergence of red spruce back to the beginning of the last glaciation of the Wisconsin era (around 110,000 years B.P.). For N_e values of 25,000 and 50,000, which are in excess of current estimates of effective population sizes in red spruce and black spruce (38), it took much more time to reach the baseline, but a time of divergence well within the Pleistocene era was still indicated. Any drastic bottleneck followed by subsequent expansion of the population shall be interpreted in light of a larger average effective population size on a geological time scale. The range of N_e values tested should include such scenarios.

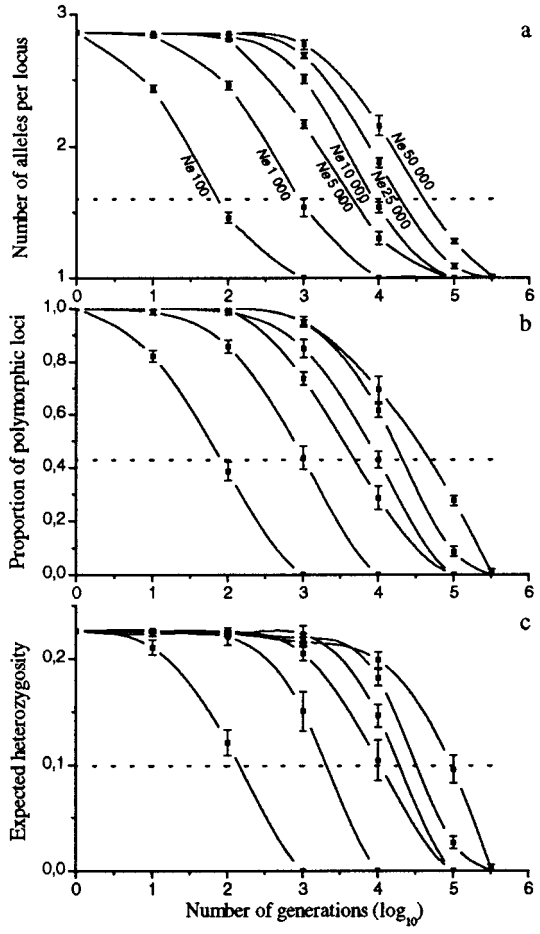

Fig. 1. Genetic drift simulations with initial allele frequencies corresponding to current values observed in the overall black spruce population at 14 polymorphic loci. N_e is the effective population size, and error bars are standard deviations from 10 replicates for each combination of effective population size and number of generations values. Dotted lines indicate the baseline levels of genetic diversity observed in allopatric red spruce.

Discussion

Several characteristics of red spruce led us to suspect that it was derived from black spruce. Successful interspecific crosses (39) and the extensive natural hybridization evident in several portions of the sympatric region (25, 40) indicate that reproductive isolation between red spruce and black spruce is weak. Also, red spruce has a smaller natural geographical range in comparison to black spruce, or most other boreal conifers (27), and it seems to be more ecologically restricted than black spruce (27). In

addition to these observations, our results now provide direct molecular evidence of a progenitor-derivative species relationship. Specifically, in comparison to black spruce, red spruce has a distinctly reduced level of genetic diversity, with fewer alleles, of which none in the current survey are unique to red spruce. Indeed, the allelic variation in red spruce seems to be strictly a subsample of that found in its progenitor, black spruce. With the addition of these observations, red spruce and black spruce are now found to possess all of the known genetic indicators of a progenitor-derivative species pair (1, 9).

The average genetic identity observed between populations of black spruce and red spruce was similar to genetic identities estimated for other progenitor-derivative species pairs, which have been found to range from 0.78 to 0.99 in angiosperms (1, 6, 7, 41–43). On the other hand, genetic identities between congeneric species that have no presumed progenitor-derivative species relationship are generally much lower, with mean values ranging from 0.60 to 0.70 (10, 44).

Although red spruce is genetically similar to black spruce and is actively hybridizing with black spruce in the area of sympatry (25, 39), every means of comparison indicates that, in contrast, it is genetically depauperate. The level of polymorphism in populations of red spruce was much lower than it was in populations of black spruce, especially in areas of allopatry with red spruce having about half the percentage of polymorphic loci than that detected in black spruce. Similar or smaller differences have been reported for derivative and progenitor species pairs in angiosperms (3, 5–8, 11, 12, 42). Heterozygosity also displayed a similar pattern, being much lower in red spruce than in black spruce, a trend similar to that reported in the angiosperm genus *Cirsium* (43). The number of alleles detected in populations of red spruce was considerably smaller than that in black spruce populations. This difference was generally more pronounced than that observed between derivative and progenitor taxa in angiosperms (3, 5–8, 12, 43, 44), and it is a key feature of a progenitor-derivative relationship (1, 9).

The genetic variation found in red spruce essentially seemed to be a subset of that observed in black spruce. Although allopatric black spruce populations had 16 unique alleles, no unique alleles were found in red spruce at the set of 26 loci surveyed, in either the sympatric or the allopatric regions. Furthermore, the most common alleles of black spruce were fixed, or nearly fixed, in red spruce at 10 of 14 polymorphic loci. Generally, derivative species have been reported to have fewer unique alleles than their progenitors (5, 43, 45).

Because natural introgressive hybridization between the two taxa is quite extensive in the region where their ranges overlap (25, 40), an appropriate protocol had to be implemented for excluding putative hybrids/introgressants at the study's entry level to estimate more precisely the genetic background specific to each taxon. The procedure followed was very effective at eliminating many of the trees sampled, especially from populations of the area of sympatry (Table 1). Despite this cautious approach, the red spruce trees retained from the area of sympatry had increased values of genetic diversity parameters and higher genetic identity with black spruce in comparison to their allopatric counterpart, indicating that traces of interpecific gene leakage still remained. This observation lends support to our separate treatment of the trees from the sympatric and allopatric areas, especially to establish the baseline level of genetic diversity for red spruce.

In contrast to red spruce, other spruce species, when analyzed by using the same set of STS markers, have been found to harbor higher levels of genetic diversity, similar to that observed in black spruce. They also have unique alleles and are polymorphic at loci at which no variation has been observed in black spruce. This trend has been shown for both the sympatric *P. glauca* (46) and the allopatric European *Picea abies* (47), indicating that the reduced genetic diversity of red spruce may be unusual in the genus *Picea*. It also illustrates that it is exceptional for the observed genetic variation to be strictly a subset of the genetic diversity found in black spruce. Unique alleles, such as those observed in black spruce and the phylogenetically more remote *P. glauca* and *P. abies* (46, 47), are likely to emerge from long-term independent evolution. The lack of unique alleles in red spruce suggests that the time of divergence from black spruce was recent on the geological time scale. This observation is reinforced by the notion of weak reproductive isolation between red spruce and black spruce (39).

Whether the divergence of red spruce is recent enough to have occurred during the Pleistocene era is a worthwhile question for our understanding of conifer biogeography and evolution. Although Pleistocene biogeographical data are scarce for boreal tree species, glaciation was prevalent during this era, with 18 to 20 advances of the ice sheet and interglacial periods accounting for about 10% of the time (48). It has already been suggested that the reduction of genetic diversity in red spruce may be explained by bottleneck effects incurred during this period (29, 30). However, it is unlikely that such bottlenecks acting alone on an old preexisting red spruce would have sculpted its genetic diversity so as to create a strict subset of the genetic diversity found in black spruce. Rather, bottleneck-induced loss of gene diversity in a preexisting red spruce would be more random, with some unique alleles of varying frequencies being retained.

If bottlenecks were experienced during the Pleistocene era, they would have been more likely for boreal conifers such as black spruce, which would have had glacial refugia located in the Appalachians or in the Mississippi valley, much further south than the current meridional limit of its natural range (48). Within the large range of parameter values tested, simulation results support this scenario of a progenitor black spruce population losing genetic diversity down to levels currently observed in red spruce, by virtue of genetic drift acting on an isolated but rather large ancestral population. Indeed, the simulation results indicated that the time since any of the major glacier advances of the Pleistocene era, such as the most recent glaciation of the Wisconsin era, would have been long enough to allow for a reduction of the genetic diversity of an isolated black spruce population to levels currently observed in red spruce, even in presence of a mild bottleneck ($N_e > 5{,}000$). Without incoming gene flow and with an effective population size of 5,000 or 10,000, which is in the same order of magnitude as that currently estimated for black spruce or red spruce populations (38), the number of generations necessary to reach the red spruce baseline level of genetic diversity was as small as 10,000, corresponding roughly to the beginning of the Wisconsin ice age if a generation time of 10 years is assumed. These results suggest that a rather rapid differentiation process may have occurred, supporting the notion of a recent divergence of red spruce on the geological time scale.

Simulation results also indicated that stronger bottlenecks are uncalled and would have quickly led to a total loss of genetic diversity within the length of time of any major glaciation of the Pleistocene era (48). Periodical population expansion during some of the short interglacial periods is likely, thus retarding the loss of genetic diversity by virtue of the maintenance of a larger effective population size on the geological time scale. Our large range of population size values tested should include such scenarios, but genetic enrichment through secondary contacts with black spruce during interglacial periods seems unlikely. Indeed, only a small directional introgression of black spruce alleles within southernmost red spruce populations has been observed since their Holocene era contact (25), suggesting that any presumed interspecific gene flow during previous interglacial periods of duration similar to that of the Holocene era would have been rather ineffective in restoring the genetic diversity of

red spruce. Such previous interglacial periods have been estimated at no more than 20,000 years (48). The inverted patterns of allele frequencies observed between red spruce and black spruce at some loci (notably *Sb01*, *Sb62,* and *Sb70)*, which may be accounted for by drift effects (in our simulations, such inverted patterns were not infrequent), add further support to the notion that the present Holocene era contact between the two species has had a rather limited homogenizing effect on their genetic background.

With all of the genetic and simulation evidence at hand, it seems likely that a single recent vicariance event of the Pleistocene era, such as the glaciation of the Wisconsin, may have promoted the accidental fragmentation and subsequent isolation of an ancestral and rather large population of black spruce into a distinct glacial refuge, allowing genetic drift and ensuing allopatric speciation of a derivative red spruce in a relatively short period. In contrast, and contrary to the simulation results and the genetic evidence at hand, to argue in favor of a more ancient, pre-Pleistocene speciation of red spruce in absence of vicariance, one would expect to observe more unique alleles arising from the accumulation of mutations over time. In addition, quantum speciation from marginal populations of black spruce would have to be invoked, which is unlikely for a species known for its outcrossing mating system and high gene flow among populations (31, 38).

It remains to be seen whether additional progenitor-derivative relationships will be identified in other conifer species complexes. Several species pairs, such as the genetically diverse *P. glauca* and the genetically less diverse *Picea sitchensis* (18, 19), or more notably, the genetically diverse *Pinus contorta* and the genetically less diverse *Pinus banksiana* (20–22), are good candidates, because they are known to hybridize naturally. Furthermore, because cases of allopatric progenitor-derivative species pairs are not unprecedented in angiosperms (e.g., ref. 6), it may be worthwhile to conduct more thorough phylogenetic studies to identify possible progenitors of genetically depauperate species of conifers, such as *Pinus resinosa* (14, 15), that are not known to hybridize with other taxa within the limits of their current natural ranges. The recognition of a well supported case of recent progenitor-derivative relationship between black spruce and red spruce offers an alternative perspective from which to reconsider some of the differences in genetic diversity found among closely related conifer taxa.

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