Genetic diversity, mating system, and conservation of a Mexican subalpine relict, *Picea mexicana* Martínez

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Abstract

Mexican spruce (*Picea mexicana* Martínez), an endangered species of the highest sky islands in México's Sierra Madre Oriental and Sierra Madre Occidental, is threatened by fire, grazing, and global warming. Its conservation depends on whether it also is threatened by inbreeding and loss of genic diversity. We used 18 isozyme markers in 12 enzyme systems to assay genic diversity, characterize the mating system, and test for recent bottlenecks in three known populations. Unbiased, expected heterozygosity (H_e) averaged 0.125. Despite a separation of 676 km between populations in the Sierra Madre Oriental and the Sierra Madre Occidental, Wright's F_{ST}, the proportion of total genic diversity among populations, was only 6.9%. Nei's genetic distance was 0.001 between the populations in the Sierra Madre Oriental and more than an order of magnitude greater, 0.019, between the Sierra Madre Oriental and Sierra Madre Occidental. However, both values point to relatively recent divergence. Mating systems were predominantly outcrossing, but with significant selfing. Multilocus estimates of selfing varied from 19% to 41%, and the means of single-locus estimates were higher, suggesting that additional inbreeding occurred by mating among relatives. Despite significant inbreeding, observed heterozygosity was as high as or higher than H_e; Wright's fixation index, F_{IS}, was -0.107. Under the observed level of selfing, positive values of F_{IS} were expected. Therefore, selection against inbreds and homozygotes must be intense. Cornuet-Luikart tests indicate recent bottlenecks in at least two of the three populations. The results suggest that Mexican spruce is a genetically viable species, and threats are primarily environmental.

Introduction

Small populations are subject to inbreeding, suffer loss of genetic diversity, and are especially susceptible to extinction (Saccheri et al. 1998; Westemeier et al. 1998). In normally outcrossing species, inbreeding depression reduces reproductive capacity and begins the spiral to extinction, an "extinction vortex" (Gilpin and Soulé 1986). Fragmentation often precedes the process by reducing or eliminating gene flow and preventing the replacement of alleles lost by random genetic drift in isolated populations. Nevertheless, some species have managed to survive fragmenta-

tion, inbreeding, and the loss of genetic diversity during extreme bottlenecks, and expand from glacial refugia to occupy extensive ranges. Possible examples are red pine (*Pinus resinosa* Ait.; Fowler and Morris 1977) and western red-cedar (*Thuja plicata* D. Don; Yeh 1988). Other species, for example bristlecone pine (*Pinus longaeva* D. K. Bailey), flourish during glacial periods and retreat to high elevation refugia during interglacials (Critchfield 1984). Mexican spruce (*Picea mexicana* Martínez) appears to be one of the latter.

Mexican spruce has a fragmented range and small populations isolated on sky islands in the Sierra Madre

Oriental and the Sierra Madre Occidental. Mexican spruce may decline to extinction if current projections of global warming materialize, because it cannot retreat northward or higher in elevation. Furthermore, species in the genus *Picea* L. are predominantly outcrossing, and inbreeding leads to substantial loss in survival and reproductive capacity (Franklin 1970) and, therefore, Mexican spruce in interglacial refugia could well be threatened by genetic stochasticity. Environmental threats to the species' survival are great, and inbreeding would increase the risk of extinction. It is of practical importance for conservation to know whether Mexican spruce is in a genetic extinction vortex.

Mexican spruce was classed as an endangered species by the IUCN (Farjon and Page 1999) based on incomplete information; i.e., that it occurred in only two populations and that one of these was almost eliminated 25 years ago (Rushforth 1986). The type locality of Mexican spruce was on the slopes of Sierra la Marta, the highest peak in México's Sierra Madre Oriental. Its only other occurrence was believed to be 676 km away on Cerro Mohinora, the highest peak of the Sierra Madre Occidental in Chihuahua. However, it occurs in at least a third location, Sierra el Coahuilón, 5 km from Sierra la Marta (Table 1; Ledig et al. 2000a).

The type locality of Mexican spruce was destroyed by fire in 1975, and botanists believed that almost all the spruce on Sierra la Marta had been lost except for a few trees (e.g., Rushforth 1986; Gordon 1991). We estimate that there may still be 1000 to 2000 spruces >10 cm diameter-breast-high on the upper slopes of Sierra la Marta, although the spruces at lower elevation are gone. We base this guess on Capó et al. (1997), who measured the density of mature spruce trees in two circular plots of 1000 m^2 on Sierra la Marta and found they averaged 300 ha^{-1}. They did not report the total area covered by spruce, but the stand is about 0.5 km across (based on our GPS readings, not corrected for satellite signal degrade, which was still imposed by the U.S. Department of Defense at the date of our observation). Our guess of population size on Sierra el Coahuilón is a few thousand trees, and on Cerro Mohinora, several hundred.

The populations on Sierra el Coahuilón and Cerro Mohinora, like that on Sierra la Marta, also have been threatened. In 1996, a fire began on Sierra el Coahuilón, but we have not assessed the extent of damage, if any, to the spruce. Cerro Mohinora had become much more accessible between our first visit

in 1985 and our most recent in 1997, and changes were obvious; the onset of grazing had reduced all spruce regeneration to heavily browsed shrubs.

Mexican spruce was discovered by botanists in 1961 on Sierra la Marta on the border of Nuevo León and Coahuila (Martínez 1961). Sierra la Marta reaches an elevation of about 3700 m, and has a flora with holarctic or boreal affiliations (Capó et al. 1997). Alpine vegetation in México represents only 0.2% of the country and includes many endemics (Bye 1993). In 1960, spruce also had been reported on the highest peak in Chihuahua, Cerro Mohinora (Correll 1960), but misidentified and not botanically described. Cerro Mohinora is about 3300 m in elevation. The spruce on Cerro Mohinora have been known as *Picea "hybrida"* (Taylor and Patterson 1980) or, locally, *Picea "indeterminada"* (J Sánchez-Cordova, pers. comm. 1988). The population at 3400 m on Sierra el Coahuilón in the Sierra Madre Oriental was only recently reported despite its proximity to Sierra la Marta (Ledig et al. 2000a). Our aerial reconnaissance suggests that spruce also occur on Sierra Potrero de Abrego, a high and relatively inaccessible ridge 9 km to the north of Sierra el Coahuilón.

Mexican spruce bears obvious similarities to Engelmann spruce (*Picea engelmannii* Parry ex Engelm.) which reaches its southern limit in the Chiricahua Mountains of southern Arizona, about 1000 km distant from Sierra la Marta. Using morphologic, phenolic, and terpenoid data and multivariate techniques, Taylor et al. (1994) found that the trees on Cerro Mohinora clustered with Mexican spruce on Sierra la Marta, and reduced both to a variety of Engelmann spruce; i.e., *P. engelmannii* Parry var. *mexicana* (Martínez) Silba. However, some individuals on Cerro Mohinora deviated widely from the centroid and may represent hybrids with blue spruce (*P. pungens* Engelm.) or Chihuahua spruce (*Picea chihuahuana* Martínez). The nearest Chihuahua spruce are about 25 km distant from Cerro Mohinora and the nearest blue spruce and Engelmann spruce are, respectively, 800 km and 700 km north in Arizona. It seems likely that the spruces in the Sierra Madre Occidental and the Sierra Madre Oriental are both relictual derivatives of Engelmann spruce, stranded on the highest peaks of their respective mountain ranges by the warming climate of the current interglacial. However, isozyme differences between Mexican spruce and Engelmann spruce are substantial (FT Ledig et al. unpublished data), and for the present, we feel it best to refer to Mexican spruce as *Picea mexicana* Martínez.

Table 1. Location of Mexican spruce stands in the States of Nuevo León, Coahuila, and Chihuahua, México

Stand	Property	Municipio[1]	Map[2]	Latitude[3]	Longitude[3]	Elev. (m)[4]
Sierra la Marta[5]	Private Property Familia Sanchez de al Peña	Rayones, N.L.	San Rafael G14C45	25°11′57″	100°21′48″	3500
Sierra el Coahuilón	Ejido[6] Nuncio	Arteaga, Coah.	San Rafael G14C45	25°14′49″	100°21′12″	3470
El Mohinora	Private Property El Venadito	Guadalupe y Calvo, Ch.	Mohinora G13C13	25°57′42″	107°02′21″	3185

[1] A municipio is a political division of a state.
[2] Number and key of the 1:50,000 national topographic maps of the Estados Unidos Mexicanos, published by the Departamento Cartografico, Secretaría de la Defensa Nacional or the Instituto Nacional de Estadística Geografía e Informatica (INEGI).
[3] In degrees, minutes, and seconds.
[4] Recorded by GPS at the center of the stand and rounded to the nearest 5 meters.
[5] Synonyms: Sierra de la Martha (Taylor and Patterson 1980; Taylor et al. 1994; but in Nuevo León, not in Coahuila as reported there); El Carmen (Martínez 1961); La Carmen (Rushforth 1986).
[6] Ejidos are lands given to groups of peasants after the 1910 revolution and usually held communally.

Mexican spruce is certainly in danger of extinction from external factors, such as fire, grazing and global warming, but whether it is in a genetic extinction spiral is not known. Nothing is known about genic diversity or outcrossing rates in Mexican spruce. Neither are we certain whether these populations are at their nadir or whether they suffered even worse bottlenecks in the past, for example during the mid-Holocene, in the warm period known in northern North America as the Xerothermic. Our objectives were to evaluate the level and distribution of genic diversity as an aid in the choice of conservation strategies for Mexican spruce. We undertook to estimate the rate of outcrossing, and its complement, selfing, to determine whether inbreeding might be a problem in the regeneration of this endangered taxon, and we tested for evidence of recent bottlenecks more severe than suggested by present population sizes.

Materials and methods

We surveyed the known stands of Mexican spruce several times over a period of 12 years for the opportunity to collect seed-bearing cones. Cone crops were infrequent to rare. Because of the paucity of cone crops, collections were spread over an extended period: the Centro de Genética Forestal (CGF) collected cones from 24 trees on Cerro Mohinora in 1988; the Universidad Autónoma Agraria Antonio Narro (UAAAN) collected cones from seven trees on Sierra la Marta and 22 trees on Sierra el Coahuilón in 1993; and the Colegio de Postgraduados collected cones from 29 trees on Sierra la Marta in 1997. We

inferred from their genotypes that none of the seven trees in the 1993 collection from Sierra la Marta were included in the 1997 collection of 29 trees, so the two collections were combined for a total sample of 36 trees.

Cones were maintained separately by trees within populations, and seeds were extracted at UAAAN or CGF and stored under refrigeration until shipped to the Institute of Forest Genetics (IFG), California. Isozymes were analyzed at IFG in 1989 (Cerro Mohinora), 1998 (Sierra la Marta and Sierra el Coahuilón), and 2000 (mating system estimates for Sierra la Marta), using starch gel electrophoresis of the megagametophytes and embryos.

In pines, the nutritive tissue of the seed is a haploid megagametophyte that gives rise to, and is of the same genotype as, the egg. Alleles at a locus can be detected by segregation among megagametophytes from a heterozygote. The genotype of the seed parent can be determined by analyzing a number of mega-gametophytes. When two different alleles at a locus are detected, the seed parent is unequivocally a heterozygote. When only one allele is detected, the tree is classified as a homozygote, although the possibility remains that it is a heterozygote and by chance the sampled seeds included only one allele. The probability of misclassification decreases with increase in sample size. We assayed at least six megagameto-phytes per tree for Cerro Mohinora, and the averages for Sierra el Coahuilón and Sierra la Marta were about 10 and 15, respectively. With a sample of six, there is a probability of 0.03125 of misclassifying a heterozygote as a homozygote. That is, the proba-

bility that all six megagametophytes in a sample from a heterozygous tree carry the same allele is $2(^1/_2)^6 = 0.03125$.

Seeds were germinated in petri dishes, and when radicles emerged, megagametophytes and embryos were dissected, separated, and extracted. For estimating allele frequencies, the number of parent trees per population, N, varied from 22 to 36 (or 2N = 44 to 72 genomes). For mating system analysis, the number of megagametophyte-embryo pairs, depending on locus, ranged from 137 to 159 in 24 progeny arrays for Cerro Mohinora, from 201 to 223 in 22 progeny arrays for Sierra el Coahuilón, and from 384 to 427 in 27 progeny arrays for Sierra la Marta.

We used techniques of starch gel electrophoresis based on the laboratory manual of Conkle et al. (1982) to assay enzyme systems. In the megagametophytes, we were able to consistently score 18 presumptive loci in 12 enzyme systems in all three populations. The enzymes were AAT, ACO, CAT, FEST, GPI, GDH, IDH, LAP, MDH, PGM, 6PG, and SKD. Most of the results presented here are based on the 18 loci scored in all three populations. However, mating system and bottleneck analyses (see below) for Sierra la Marta and Sierra el Coahuilón employed additional loci in the TPI and UGP systems because our repertoire expanded over the course of the study. ALD was also consistent for Sierra la Marta and Sierra el Coahuilón. We interpreted the number of loci and alleles by drawing on the experience gained in our laboratory from studies of allozymes of other conifer species (Conkle 1981). Samples of red pine were included on each gel to aid interpretation.

We estimated percent polymorphic loci, alleles per locus, heterozygosity, and Nei's (1978) unbiased genetic distance with BIOSYS (Swofford and Selander 1981). For small samples such as ours, BIOSYS calculates unbiased heterozygosity (Nei 1978). Fixation indices (**F**) within populations were calculated as the mean deviation of loci from Hardy-Weinberg expectations. We also used BIOSYS to calculate Wright's (1965) F-statistics. BIOSYS calculates $\mathbf{F_{IS}}$ and $\mathbf{F_{IT}}$, the fixation indices of trees relative to the population and the meta-population, respectively, as weighted averages across alleles. $\mathbf{F_{ST}}$, the proportion of the total genic diversity among populations, is calculated from the relationship: $\mathbf{1 - F_{IT} = (1 - F_{IS})(1 - F_{ST})}$. All inferences apply to the population of mature, cone-bearing trees.

The degree of genetic isolation among populations was estimated by **Nm**. **Nm** is the number of migrants per generation. **Nm** was calculated from Wright (1951): $\mathbf{Nm = (1 - F_{ST})/4F_{ST}}$.

We used the computer program BOTTLENECK, described by Cornuet and Luikart (1996) to determine whether effective population numbers had been restricted in the recent past. The infinite allele model (Kimura and Crow 1964) was chosen because empirically it tends to fit allozyme data better than alternatives (Luikart and Cornuet 1998). The Wilcoxon sign-rank test was preferred to the sign test because the former has higher power and can be used with as few as four polymorphic loci (Piry et al. 1999). Six polymorphic loci were available for Cerro Mohinora, eight for Sierra el Coahuilón, and ten for Sierra la Marta.

For mating system analysis, we used Ritland's (1986, 1989, 1990a, 1994) MLTR and MLTF programs to calculate the single- and multiple-locus estimates of outcrossing rate, $\mathbf{t_s}$ and $\mathbf{t_m}$, at the population level. MLTF was used to estimate $\mathbf{t_m}$ because the probability of outcrossing becomes much more accurate when information on the megagametophyte genotype is used (Ritland 1990a). Knowing the contribution of the egg (the haploid genotype of the megagametophyte) to the embryo, the pollen contribution can be deduced by subtraction so that smaller progeny arrays are adequate and bias is reduced. The Newton-Raphson method was used for iteration, and **p**, the allele frequency, and **t** were estimated jointly. Standard errors for $\mathbf{t_s}$ at one locus were taken from the estimated values in 500 bootstraps using MLTR. MLTR was also used to estimate the correlation of outcrossed paternity (**rp**) among progeny within a family.

Results

Of the 18 loci scored in all three populations, 8 were polymorphic in at least one population (Table 2), and ten (CAT-1, FEST, GDH, AAT-1, AAT-2, IDH-1, MDH-1, MDH-2, GPI-2, and SKD-2) were monomorphic in all three. TPI-1 and UGP-1 were not scored in Cerro Mohinora, but were polymorphic in Sierra la Marta and Sierra el Coahuilón. ALD1, also scored in Sierra la Marta and Sierra el Coahuilón but not in Cerro Mohinora, was monomorphic. The common allele at a locus in one population was usually the common allele in the others, except for PGM-1, where allele-2 was most common at Cerro Mohinora but in relatively low frequency at Sierra la Marta and Sierra el Coahuilón.

Table 2. Allele frequencies for 8 polymorphic loci in three populations of Mexican spruce

Locus/ allele		Population[1] (sample size[2])		
		CM (48)	SLM (72)	SEC (44)
AAT-3/	1	0.250	0.486	0.361
	2	0.750	0.514	0.639
ACO-1/	1	0.522	0.524	0.711
	2	0.478	0.476	0.289
LAP-1/	1	1.000	0.972	0.932
	2	0.000	0.028	0.068
LAP-2/	1	1.000	0.861	0.932
	2	0.000	0.139	0.068
MDH-3/	1	0.479	0.389	0.364
	2	0.125	0.181	0.182
	3	0.396	0.431	0.455
6PG-2/	1	0.979	0.986	1.000
	2	0.021	0.014	0.000
PGM-1/	1	0.333	0.792	0.818
	2	0.667	0.208	0.182
SKD-1/	1	0.792	1.000	1.000
	3	0.208	0.000	0.000

[1] CM = Cerro Mohinora, SLM = Sierra la Marta, SEC = Sierra el Coahuilón.
[2] 2N, the number of genomes, or twice the number of trees.

Unbiased estimates of expected heterozygosity, H_e, for the 18 loci scored in all three populations were in a narrow range, from 0.117 to 0.130 (Table 3). The percent polymorphic loci, **P**, ranged from 33.3% to 38.9%, and the number of alleles per locus, **A**, was 1.4 in all three populations. Observed heterozygosity was about equal to expected heterozygosity in Cerro Mohinora and greater than expected in Sierra la Marta and Sierra el Coahuilón (Table 3). The mean of the inbreeding coefficients, −0.094, was similar to Wright's F_{IS}, −0.107 (Table 4). A negative **F** indicates heterozygote excess and suggests selection for heterozygotes.

Wright's F_{ST} was 0.069 ± 0.027; i.e., 6.9% of the genic diversity is among populations (Table 4). However, much of this variation was a result of the inclusion of Cerro Mohinora. F_{ST} was only 0.013 between Sierra la Marta and Sierra el Coahuilón, but was 0.068 and 0.074 between Cerro Mohinora and Sierra la Marta and Sierra el Coahuilón, respectively. The number of migrants per generation, **Nm**, estimated from Wright's F_{ST} was 19.0 between Sierra la Marta and Sierra el Coahuilón, and 3.1 or 3.4 between these populations and Cerro Mohinora.

Nei's genetic distance (**D**) was minimal between Sierra la Marta and Sierra el Coahuilón and an order of magnitude greater between either of these two populations and Cerro Mohinora (Table 5).

The Wilcoxon sign-rank test indicated greater heterozygosity on Cerro Mohinora and Sierra el Coahuilón than that expected for populations at mutation-drift equilibrium (Table 6), suggesting that they have been bottlenecked no more than a few generations ago. Results for Sierra la Marta, with a Wilcoxon sign-rank probability of 0.08, bordered on statistical significance.

The multilocus outcrossing rates at the population level ranged from 0.590 (0.516 < t_m < 0.664; $P = 0.95$) at Sierra la Marta to 0.807 (0.709 < t_m < 0.905; $P = 0.95$) at Sierra el Coahuilón (Table 7). All three populations exhibit significant selfing. In every case, the mean single-locus outcrossing rate was lower than the multilocus rate, but the differences were within two standard errors. Nevertheless, the consistent differences between estimates of t_m and t_s suggest that some inbreeding occurred by crosses among relatives, in addition to inbreeding by selfing. The correlation of outcrossed paternity, **rp**, among progeny within a family were exceptionally high, which suggests a high proportion of full-sibs within a maternal family. Estimates of t_s were heterogeneous among loci at Cerro Mohinora and Sierra la Marta, suggesting selection on the allozymes or that they are in disequilibrium with loci under selection.

Discussion

The estimates of genic diversity in Mexican spruce were higher than average for long-lived, woody endemics and close to average for outcrossing endemic plant species in general; mean expected heterozygosity (H_e) was 0.056 for 20 long-lived woody endemics (Hamrick et al. 1992) and 0.142 for 57 outcrossing endemic plant species (Hamrick and Godt 1996). For the Mexican spruce data set (18 loci), unbiased H_e averaged 0.125. H_e in Mexican spruce was about 25% lower than values for gymnosperms in general (0.151) or outcrossing, wind-pollinated trees (0.154) reviewed by Hamrick et al. (1992). However, the mean heterozygosity for ten populations of Chihuahua spruce, a species endemic to the Sierra Madre Occidental, was only 0.093 and only the

Table 3. Genic diversity and fixation indices in Mexican spruce: H_e – expected heterozygosity (unbiased estimate), H_o – observed heterozygosity, **P** – percent polymorphic loci, **A** – number of alleles per locus, **F** – fixation index[1], and F_e – equilibrium inbreeding coefficient, calculated from the outcrossing rate (standard errors in parentheses)

Population	N[2]	H_e	H_o	P	A	F	F_e
Cerro Mohinora	23.9	0.130 (0.051)	0.124 (0.049)	33.3	1.4 (0.1)	0.007	0.223
Sierra la Marta	35.2	0.129 (0.051)	0.147 (0.061)	38.9	1.4 (0.1)	−0.103	0.258
Sierra el Coahuilón	21.6	0.117 (0.048)	0.137 (0.056)	33.3	1.4 (0.1)	−0.185	0.107
Mean		0.125	0.136	35.2	1.4		

[1]Calculated as the mean of the fixation indices for all polymorphic loci.
[2]Mean number of trees per locus.

Table 4. Estimates of Wright's (1965) F-statistics for 8 polymorphic loci in Mexican spruce

Locus	F_{IS}	F_{IT}	F_{ST}
AAT1	−0.310	−0.257	0.040
ACO3	−0.192	−0.154	0.032
LAP1	−0.060	−0.033	0.025
LAP2	−0.131	−0.074	0.050
MDH3	−0.046	−0.039	0.006
6PG2	−0.018	−0.012	0.007
PGM1	0.116	0.308	0.217
SKD1	−0.011	0.140	0.149
Mean	−0.107	−0.030	0.069

Table 5. Half-matrix of Nei's (1978) unbiased genetic distances calculated from frequencies of 18 loci scored in megagametophytes (**D**, above diagonal) and geographic distances (in km, below diagonal) between pairs of Mexican spruce populations

Population[1]	CM	SLM	SEC
CM		0.019	0.019
SLM	676		0.001
SEC	676	5	

[1]CM = Cerro Mohinora, SLM = Sierra la Marta, SEC = Sierra el Coahuilón.

largest population of Chihuahua spruce had as high a heterozygosity as Mexican spruce on Cerro Mohinora or Sierra la Marta (Ledig et al. 1997). Heterozygosity for two populations of Martínez spruce (*Picea martínezii* T.F. Patterson), a very rare endemic of the Sierra Madre Oriental, was 0.101 and 0.121, similar to that for Mexican spruce (Ledig et al. 2000b). Percent polymorphic loci (**P**) in Mexican spruce was about 35%, which is less than the mean for outcrossing endemics (54.4%) but slightly higher than that for woody endemics (26.3%) reviewed by Hamrick and Godt (1996) and Hamrick et al. (1992).

Diversity was not related to population size. Although we have no census data, the distance between GPS readings taken at the extremes of the stands indicate that Sierra el Coahuilón, with the lowest H_e, is the largest in area, extending over 3 km along and just below the ridge line. The distance between the edges of the populations on Cerro Mohinora and Sierra la Marta are about 1 km and 0.5 km, respectively. However, the population on Sierra la Marta had a much larger extent before the disastrous fire of 1975.

Mexican spruce had few rare alleles and most polymorphic loci had only two alleles, suggesting past bottlenecks. The excess of heterozygosity at Hardy-Weinberg expectations over that in populations at mutation-drift equilibrium, in fact, indicated recent reductions in effective population number. However, loci not in Hardy-Weinberg proportions may bias the test, and most of the loci in our samples had excess heterozygosity. Nevertheless, excluding data not in Hardy-Weinberg proportions did not change the results in tests of several data sets (Luikart and Cornuet 1998), so the importance of the bias is uncertain. It is noteworthy that the two populations for which evidence of past bottlenecks is strongest, Cerro Mohinora and Sierra el Coahuilón, are the two lowest in elevation. In a warming climate, spruce could retreat further upslope at Sierra la Marta than at either of the two other locations and, therefore, suffer less of a reduction in size.

Genetic structure of Mexican spruce was less pronounced than expected for endemics with fragmented ranges. About 6.9% of the observed diversity was among populations, compared to 17.9% for all outcrossing plant endemics (Hamrick and Godt 1996) and 14.1% for woody endemics (Hamrick et al. 1992). In Chihuahua spruce, about 25% of the

Table 6. Cornuet and Luikart (1998) test for recent bottlenecks in Mexican spruce populations under the infinite allele model of mutation-drift equilibrium for neutral alleles

Locus	Cerro Mohinora				Sierra el Coahuilón				Sierra la Marta			
	n^1	k^2	H_e^3	H_{eq}^4	n	k	H_e	H_{eq}	n	k	H_e	H_{eq}
ACO-1	46	2	0.510	0.226	38	2	0.422	0.228	42	2	0.511	0.222
GOT-3	48	2	0.383	0.226	36	2	0.475	0.231	72	2	0.507	0.203
LAP-1	–	–	–	–	44	2	0.130	0.211	72	2	0.055	0.204
LAP-2	–	–	–	–	44	2	0.130	0.233	72	2	0.243	0.206
MDH-3	48	3	0.611	0.368	44	3	0.642	0.384	72	3	0.639	0.351
6PG-2	48	2	0.042	0.214	–	–	–	–	72	2	0.028	0.209
PGI-1	–	–	–	–	–	–	–	–	54	2	0.108	0.214
PGM-1	48	2	0.454	0.222	44	2	0.305	0.222	72	2	0.334	0.200
SKD-1	48	2	0.336	0.206	–	–	–	–	–	–	–	–
TPI-1	–	–	–	–	44	2	0.406	0.214	72	2	0.503	0.206
UGP-1	–	–	–	–	44	2	0.359	0.229	72	2	0.317	0.199
Prob.[5]		0.039				0.037				0.080		

[1] Number of genomes sampled.
[2] Number of alleles observed.
[3] Expected heterozygosity under Hardy-Weinberg.
[4] Expected heterozygosity under infinite allele model of mutation-drift equilibrium (Kimura and Crow 1964).
[5] Wilcoxon sign-rank test one-tailed probability for heterozygosity excess.

genetic diversity was among populations. In Martínez spruce, only 2.4% of the total diversity was between the two most widely separated populations, 147 km apart. Most of the variation in Mexican spruce was between Cerro Mohinora in Chihuahua and Sierra el Coahuilón and Sierra la Marta in Nuevo León.

Nei's (1978) genetic distance (**D**) can be used to bracket the time (**T**) since populations began to diverge and, therefore, date the time since the spruce on Cerro Mohinora separated from those in the Sierra Madre Oriental: $\mathbf{T = D/2\alpha}$, where α is the mutation rate. For an assumed α of 10^{-5} and 10^{-6} and a genetic distance of 0.019 (Table 5), the estimate of **T** seems very low, 950 to 9500 yrs.

The history of spruce in México suggest, in fact, that they were recently more widespread and a warming climate was probably responsible for their decline. Spruce in México occurred at least as far south as the Isthmus of Tehuantepec (18°09′ N) in the mid-Pliocene, 5 million years ago (Graham 1993). Pollen in the ancient bed of Lake Texcoco, now largely beneath México City, and in Lake Chalco in the basin of México (19°28′ N) show that spruce occurred in the surrounding uplands of the Transverse Volcanic Belt at the end of the Pleistocene (Clisby and Sears 1955) and at least as recently as 7000 to 8000 yr before present (B.P.; Lozano-García et al. 1993, MdS Lozano-García, pers. comm. 1997). All spruce taxa in México are now restricted

Table 7. Single locus and multilocus outcrossing rates[1] at the population level in Mexican spruce (standard errors in parentheses)

Locus	Cerro Mohinora	Sierra la Marta	Sierra el Coahuilón
ACO-1	0.089 (0.051)	–	–
IDH-2	–	0.383 (0.101)[2]	–
LAP-1	–	–	0.776 (0.166)
LAP-2	–	0.746 (0.074)	0.862 (0.107)
MDH-3	0.728 (0.079)	0.518 (0.051)	0.744 (0.073)
PGM-1	–	0.578 (0.066)	0.647 (0.120)
6PG-1	–	0.726 (0.099)	–
SKD-1	0.808 (0.126)	–	–
TPI-1	–	–	0.846 (0.131)
UGP-1	–	0.450 (0.071)	0.798 (0.103)
$\bar{t}_s^2$	0.539 (0.094)	0.524 (0.058)	0.753 (0.056)
t_m	0.635 (0.056)	0.590 (0.037)	0.807 (0.049)
rp^2	0.990 (0.070)	0.465 (0.137)	0.990 (0.131)

[1] Calculated with Ritland's (1990b) MLTF unless otherwise noted.
[2] Calculated with Ritland's (1994) MLTR.

to small, isolated stands, predominantly on the north slopes of steep arroyos in the Sierra Madre Occidental (Chihuahua spruce) or in cool, moist montane forests of the Sierra Madre Oriental (Martínez spruce), or on the highest peaks of these ranges (Mexican spruce).

Whether Mexican spruce occurred as far south as México City is not known because spruce pollen cannot be identified to species level. Genetic and paleobotanic evidence suggest that some spruce taxon or taxa retreated northward coincident with the period of global warming that ended the Pleistocene glaciation (Ledig et al. 1997; Ledig et al. 2000b). If Mexican spruce occurred in the vicinity of México City, it is conceivable that it could have retreated north along both the Sierra Madre Occidental and Sierra Madre Oriental, which are separated by the arid Meseta Central. However, that begs the question of why it did not persist in the Transverse Volcanic Belt, where elevations range up to 5611 m. Moreover, McDonald (1993) argued that topography is not conducive to migration of high elevation taxa between México City and the Sierra Madre Oriental, and the high endemism of subalpine habitats in the Sierra Madre Oriental suggests that they were not linked with the Transverse Volcanic Belt during the Pleistocene. The relative lack of differentiation between Mexican spruce populations in the Sierra Madre Oriental and the Sierra Madre Occidental is surprising and cannot yet be explained satisfactorily (but see below).

Low rates of gene flow may lead to differentiation among populations because of random genetic drift, but estimates of **Nm** were 3.1 and 3.4 between Nuevo León and Chihuahua. An **Nm** of 3.1 per generation must certainly reflect past gene exchange and overestimate current levels of gene flow across a gap as wide as 676 km. **Nm** takes several hundred generations to reach equilibrium once gene flow has ceased (Slatkin and Barton 1989). Nevertheless, the estimate is valuable in comparison with other species and because it, like Nei's genetic distance, suggests that the populations were in contact within recent geological time. **Nm** in pines ranges between 3.4 and 17.2 in rangewide studies (reviewed in Ledig 1998). Lower values of **Nm** have been found in the Mexican endemic, Chihuahua spruce (**Nm** = 0.8), and in Coulter pine (*Pinus coulteri* D. Don; **Nm** = 1.3), species with highly fragmented ranges in which the evidence for genetic drift was unmistakable (Ledig et al. 1997; Ledig 2000). As might be expected, gene flow between Sierra la Marta and Sierra el Coahuilón was very high: **Nm** = 19.0.

Outcrossing predominated in all three populations, although the data suggest the highest rates of selfing in the smaller populations, Cerro Mohinora and Sierra la Marta. The rate of outcrossing was significantly higher (Table 7) in Sierra el Coahuilón. The mixed mating model for the estimation of outcrossing rate assumes loci are in linkage equilibrium. Although the number of progeny in our families was too small to estimate disequilibrium or even the recombination rate between loci, linkage among the loci that we used is probably weak, at best. Linkage arrangements are highly conserved in conifers, and among the loci we used, none were in the linkage groups reported by Conkle (1981) for five pines.

Single-locus estimates are not, of course, affected by linkage, and the means of the single-locus estimates, t_s, of outcrossing were similar to t_m, but invariably lower. Therefore, linkage probably had little effect on the estimate of t_m reported here. On the other hand, differences between single-locus and multilocus estimates of outcrossing suggest that some crossing between relatives may be occurring as a result of limited dispersal of progeny and pollination between neighbors or pairs of synchronously flowering trees. The difference between t_s and t_m approached statistical significance at Cerro Mohinora.

Selfing and/or crossing between related trees was not reflected in excess homozygosity. Quite the contrary, heterozygote excess was apparent at Sierra la Marta and Sierra el Coahuilón. The equilibrium inbreeding coefficient, F_e, is related to the outcrossing rate, t, as (Allard et al. 1968): $F_e = (1 - t)/(1 + t)$. Substituting our estimates of t_m for t yields an estimated F_e of 0.107 for Sierra el Coahuilón, 0.223 for Cerro Mohinora, and 0.258 for Sierra la Marta, compared to observed fixation indices of -0.185, 0.007, and -0.103 (Table 3), respectively. Thus, F_e does not even approximate observed F, and the difference accentuates the significance of the observed excess heterozygosity. Selection against inbreds and for heterozygotes must be intense. Balancing selection in similar environments (high alpine habitat) may stabilize gene frequencies, and is a potential explanation for the lack of population differentiation (low F_{ST} and **D**) between the Sierra Madre Occidental and the Sierra Madre Oriental. This would result in an overestimate of **Nm** and an underestimate of time since divergence.

Conclusions

Mexican spruce may not yet be in a genetic extinction vortex. Genetic diversity, based on 18 loci, is about average for plant endemics. Nor does the genetic structure of Mexican spruce reflect the estimated rate

of selfing, because heterozygosity meets or exceeds Hardy-Weinberg expectations; two of the three populations were characterized by excess heterozygosity. Although some selfing is indicated, it is not nearly as high as in Chihuahua spruce, in which rates of selfing for two small populations were 100% and 85%, or in Martínez spruce, in which one population had a rate as high as 60%.

The main threats to Mexican spruce are grazing, fire, and global warming. None of the sites (Table 1) are legally protected, and grazing has become a major threat to regeneration on Cerro Mohinora. Cerro Mohinora and, perhaps, all three sites should be declared protected areas and fenced to exclude grazing. Fire is a more serious threat and more difficult to deal with. Steps that should be initiated include the construction of wide firebreaks below the stands, education to reduce incendiary fires, and a plan that calls for early response to fires that threaten the habitats of Mexican spruce and other endangered species. The most serious threat to Mexican spruce may be global warming, because stand management can do nothing to prevent it. Already restricted to the highest sky islands in northern México, there is nowhere that Mexican spruce can migrate to escape warming temperatures. *Ex situ* conservation is the only option to offset the effects of global warming. Seeds should be collected and divided among several seedbanks for long-term storage. Cuttings should be collected and multiplied by cloning to establish field genebanks, probably in the United States or Canada, north of the present range. Cloning has been successful with Mexican spruce, and a few clones and seedlings have been established in Piceta in Ontario and British Columbia (Gordon 1990, 1991). Genetically, the situation is not yet desperate.

Acknowledgments

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