The mating system and genic diversity in Martínez spruce, an extremely rare endemic of México's Sierra Madre Oriental: an example of facultative selfing and survival in interglacial refugia

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Abstract: Martínez spruce (*Picea martínezii* T.F. Patterson) is a conifer currently passing through a bottleneck, reduced to a few relict populations totaling less than 800 trees. We used isozyme markers to analyze the mating system and survey the level of genic diversity in two populations. The mating system was characterized by a high frequency of selfing. The multilocus outcrossing rates (t_m) and 95% confidence intervals were only 0.399 ($0.197 < t_m < 0.601$) for the smallest population and 0.589 ($0.475 < t_m < 0.703$) or 0.685 ($0.465 < t_m < 0.905$), depending on year, for the largest. These are among the lowest rates of outcrossing observed in conifers. The fixation indices for the two populations were –0.058 and 0.121, less than expected for such high levels of selfing. Expected heterozygosity, unbiased H_e, based on 22 loci in 13 enzyme systems, was 0.121 and 0.101 in the two populations. The proportion of the total genic diversity between populations, F_{ST}, was 2.4%. *Nm*, the number of migrants per generation, was about 1.00 or 10.17, depending on the method of estimation. The time since the two populations were isolated was estimated from Nei's genetic distance as only 150 to 15 000 years, which is consistent with a hypothesis of population collapse during late Pleistocene or Holocene warming. We discuss the implications for conservation.

Résumé : L'épicéa de Martinez (*Picea martinezii* T.F. Patterson) est une espèce coniférienne affectée actuellement par un rétricissement marqué de son effectif, avec quelques populations reliques totalisant moins de 800 arbres. Les auteurs ont utilisé des marqueurs isoenzymatiques afin d'estimer le niveau de diversité génétique et le système d'accouplement au sein de deux populations. Le système d'accouplement était caractérisé par une fréquence élevée d'autogamie. Les taux d'allogamie multilocus (t_m) et les intervalles de confiance au niveau 95% étaient de 0,399 ($0{,}197 < t_m < 0{,}601$) pour la plus petite population et de 0,589 ($0{,}475 < t_m < 0{,}703$) et 0,685 ($0{,}465 < t_m < 0{,}905$) pour la plus grande population, dépendamment de l'année visée. Ces taux sont parmi les plus faibles observés chez les conifères. Les indices de fixation pour chacune des deux populations affichaient des valeurs de –0,058 et 0,121. Ces valeurs sont moindres que les espérances découlant de niveaux si élevés d'autogamie. L'hétérozygotie espérée non biaisée H_e, estimée à partir de 22 loci codant pour 13 systèmes enzymatiques, affichait des valeurs de 0,121 et 0,101 pour chacune des deux populations. L'indice F_{ST} indiquant la proportion de la diversité génétique totale attribuable aux différences entre populations était de 2,4%. Le nombre de migrateurs par génération, Nm, variait de 1,00 à 10,17, selon la méthode d'estimation utilisée. L'intervalle de temps depuis la séparation des deux populations a été estimé à partir de la distance génétique de Nei. Cet intervalle est faible et varie de 150 à 15 000 ans, ce qui est congruent avec l'hypothèse d'une chute marquée de l'effectif durant la fin du Pléistocène ou durant le réchauffement subséquent pendant l'Holocène. Les auteurs discutent des implications pour la conservation de l'espèce.

[Traduit par la Rédaction]

Received October 26, 1999. Accepted February 25, 2000.

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Introduction

Conifers in the family Pinaceae, which includes spruce, are categorized as strongly outcrossing (Schemske and Lande 1985) with high levels of genic diversity (Hamrick and Godt 1989). When subjected to selfing, they expose a genetic load that results in profound inbreeding depression (reviewed by Franklin 1970). Yet, a few species are devoid, or nearly devoid, of genic diversity (Fowler and Morris 1977; Ledig and Conkle 1983; Copes 1981; Yeh 1988), including red pine (*Pinus resinosa* Ait.), Torrey pine (*Pinus torreyana* Parry ex Carr.), and western redcedar (*Thuja plicata* Donn. ex D. Don). At least one of these (red pine) suffers, at most, only minor inbreeding depression when selfed (Fowler 1965).

Population bottlenecks accompanied by inbreeding and drift are usually invoked to explain the origin of anomalous conifers that lack genic diversity. Because the Pinaceae have no self-incompatibility system, selfing is possible when numbers of breeding individuals are reduced, as in refugia or after colonizing events. Therefore, they satisfy Baker's (1955) criteria for successful colonizers. The ability to colonize may be important in a predominantly cool temperate and boreal family that expands from relatively narrow refugia to migrate forth during glacial periods.

Inbreeding depression, the major barrier to selfing in the Pinaceae (Ledig 1986), exerts its first effect on seed yield. For example, selfing reduces seed yield 60% on the average in spruce (Franklin 1970). However, species in the Pinaceae have a high reproductive output. Stands of Norway spruce (*Picea abies* (L.) Karst.) in Finland produced an annual yield of 83 filled seeds/m^2, averaged over a 6- to 9-year period (Sarvas 1968), and high elevation stands of Engelmann spruce (*Picea engelmannii* Parry ex. Engelm.) in Colorado produced an average of 25.5 seeds/m^2 per year over a 10-year period (Alexander et al. 1982). Because conifers are iteroparous and long-lived, their reproductive life may extend over centuries, and total seed output could be tremendous, perhaps capable of accommodating massive losses due to inbreeding depression. Thus, facultative selfing combined with the potential for high reproductive output may enable some Pinaceae to pass the sieve of inbreeding and could have contributed to their survival over millions of years and many glacial cycles.

Martínez spruce (*Picea martínezii* T.F. Patterson) was not discovered by botanists until 1983 (Müller-Using and Alanís 1984). It was found in two localities in the Sierra Madre Oriental and was erroneously thought to be Chihuahua spruce (*Picea chihuahuana* Martínez). However, the nearest populations of Chihuahua spruce are at least 510 km distant in the Sierra Madre Occidental, separated from the Sierra Madre Oriental by the arid Meseta Central of México. Somewhat later, Patterson (1988) visited the stands in the Sierra Madre Oriental and recognized the spruce as a new species.

B. Müller-Using and S. Velázquez (Müller-Using and Lässig 1986) measured all Martínez spruce over 10 cm diameter at breast height (DBH) at its southernmost occurrence, Cañada La Tinaja in the Ejido La Encantada, Nuevo León, and at the northern location, which is at the base of a nearly 800 m tall limestone precipice, known as El Butano, near La Trinidad, Nuevo León (Table 1). The two locations are 147 km apart. They counted 65 trees at La Tinaja and 350 at El Butano. Plans were formulated to protect these rare trees from harvest (Müller-Using and Alanís 1984). However, in such small populations, inbreeding may be a problem and protection alone may be insufficient for conservation. Within the last year, we found Martínez spruce in two new areas; these stands are within 18 km of La Tinaja but at least 132 km south of El Butano. The total number of spruces in the newly discovered locations may double the census for the species. Nevertheless, the total number of mature Martínez spruce is probably only about 800.

Spruce in México was more widespread in the Pleistocene and only recently reduced in area and numbers. Pollen in the ancient bed of Lake Texcoco, which is now México City, and in Lake Chalco in the Basin of México show that spruce occurred in the surrounding uplands at the end of the Pleistocene (Clisby and Sears 1955) and at least as recently as 7000–8000 years before present (BP; Lozano-García et al. 1993; M.d.S. Lozano-García, personal communication). México City is about 500 km south of La Tinaja.

Martínez spruce exists in small refugia similar to those hypothesized as the origin of genetically depauperate species. If it was reduced in numbers as recently as the late Pleistocene or Holocene, it is unlikely to have had sufficient time to adapt to inbreeding. Martínez spruce and similar relicts may be snapshots in time, examples of populations in the incipient stage of purging their genetic load. Whether they survive depends on chance (surviving catastrophe and environmental and demographic stochasticity) and, perhaps, on whether they can purge their genetic load through inbreeding.

We surveyed genic diversity and analyzed the mating system in Martínez spruce. We collected seeds from populations of Martínez spruce at La Tinaja and El Butano, and surveyed genic diversity using isozyme electrophoresis. Joint analysis of gametophyte–embryo pairs was used to estimate the rate of outcrossing. Martínez spruce provides a likely example of a stage in the evolution of genetically depauperate northern conifers believed to have survived bottlenecks during the Pleistocene glaciation and the subsequent xerothermic, with a concomitant reduction in diversity, and in at least one case, a tolerance of inbreeding.

Materials and methods

Cones were collected at La Tinaja in October 1988. Attempts were made to sample El Butano beginning in 1985, but because of the lack of cone crops and the difficulty of access, we were unsuccessful until September 1994. An even better cone crop was collected at El Butano in 1997. The lack of cone crops and the paucity of cone-bearing trees (see below) are detailed, because they have implications for the interpretation of the results and the conservation of this rare species.

The sampled trees in each stand were widely spaced over the area occupied by the spruce. The cones were collected by climbing, and often the entire crop was removed. Our samples represent the entire population of cone-bearing Martínez spruce at La Tinaja and El Butano, the trees that will give rise to the next generation, for the years in which we attempted to collect (1985–1997). Cones were maintained separately by tree and transported to the Centro

Table 1. Location of Martínez spruce stands in the State of Nuevo León, Mexico, sampled for mating system analysis.

Stand (census)	Property	Municipio[a]	Map[b]	Latitude (N)	Longitude (W)	Elevation (m)[c]
La Tinaja (65)[d]	Ejido[e] La Encantada	Zaragoza	Zaragoza F14A17	23°53′24″	99°47′30″	2515
Cañón el Butano (350)[d]	Ejido La Trinidad	Montemorelos	Rayones G13C46	25°10′41″	100°07′37″	2180

[a] A municipio is a political division of a state.

[b] Number and key of the 1 : 50 000 national topographic maps of the Estados Unidos Mexicanos, published by the Comisión de Estudios del Territorio Nacional (CETENAL), Secretaría de la Presidencia.

[c] Elevation was determined using the global positioning system at the center of the stand and rounded to the nearest 5 m.

[d] Number of trees ≥10 cm DBH (Müller-Using and Lässig 1986).

[e] Ejidos are lands given to groups of peasants after the 1910 revolution and usually held communally.

de Genética Forestal in Chapingo, México. Seeds were extracted and stored at 1°C until needed.

The goal was to sample 35 trees, but that was not attainable because of the low frequency of trees with cones. The actual sample in 1988 (La Tinaja) and 1994 (El Butano) was 21 trees at each site. However, seed germination was low; many trees with cones failed to yield viable seeds, particularly trees at El Butano. Of the 21 trees sampled from La Tinaja, we were able to analyze seeds of only 18 trees, and of the 21 trees sampled in 1994 at El Butano, only 10 produced viable seeds, which represents 36 and 20 genomes, respectively. In 1997, we collected viable seeds from 26 trees at El Butano, representing 52 genomes.

Diploid, vegetative tissue would have provided larger sample sizes to characterize diversity. However, mating system analysis is not possible without seeds or seedlings. Mating system analysis was crucial to determine whether inbreeding was a problem in the conservation of Martínez spruce.

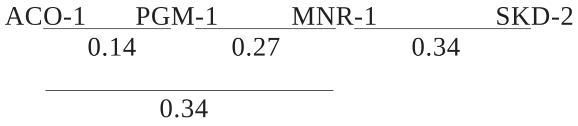
Seeds from La Tinaja were germinated for isozyme analysis in 1989, and for the two collections from El Butano, in 1995–1996 and 1998, respectively. When the radicals appeared through the seed coat, the megagametophyte and embryo were excised and separated.

Because the megagametophyte of spruce is haploid, alleles at a locus can be detected by segregation among seeds from a heterozygote. The genotype of the seed parent can be determined by analyzing a number of megagametophytes. 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. A sample of six (La Tinaja) reduces the probability of misclassifying a heterozygote as a homozygote to about 0.03. That is, the probability that all six megagametophytes in a sample from a heterozygous tree carry the same allele is $2(0.5)^6 = 0.03$. For a sample of 40 (the 1997 sample from El Butano), the probability of misclassification becomes practically nil.

We simultaneously analyzed the embryo alongside the megagametophyte to characterize the mating systems in the two populations. Knowing the contribution of the egg (the haploid genotype of the megagametophyte) to the zygote, the pollen contribution can be deduced by subtraction. We attempted to assay eight megagametophyte–embryo pairs per tree from La Tinaja; actual sample size varied, but averaged 6.4, or a total of 115 pairs. In the 1994 sample from El Butano, we attempted to assay 18 megagametophyte–embryo pairs per tree, but because of poor germination, the mean was 14.6, for a total of 146 pairs. In the 1997 sample from El Butano, we attempted to assay 40 megagametophyte-embryo pairs from each of 26 trees; the actual mean was 38.3, a total of 996 progeny. Identifying the megagametophyte genotype allows the pollen contribution to be identified unambiguously, and the estimate of outcrossing becomes much more accurate (Ritland 1990*a*).

We used the techniques of starch gel electrophoresis described by Conkle et al. (1982) to assay 13 enzyme systems. 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, an almost invariably homozygous species, were included on each gel to aid interpretation. Where several zones of activity were observed for a single enzyme, hyphenated numerals following the enzyme abbreviation were used for identification. The laboratory analyses took place over a period of 10 years, and not all loci were scorable in every analysis. However, 22 presumptive loci were consistently scored and used in the statistical analysis: ACO-1, CAT-1, CAT-2, FEST, GDH, GOT-1, GOT-2, GOT-3, IDH-1, IDH-2, LAP-1, LAP-2, MDH-2, MDH-3, MNR-1, PGI-1, PGI-2, PGM-1, 6PG-1, 6PG-2, SKD-1, and SKD-2. For the mating system analysis, three polymorphic loci were analyzed for La Tinaja and the 1994 sample from El Butano (ACO-1, MNR-1, and SKD-2) and four in the 1997 sample from El Butano (GOT-3, MNR-1, PGI-2, and SKD-2).

We calculated linkage among loci using Kosambi (1944), and combined data from different seed trees using heterogeneity chi-square (Snedecor 1956, pp. 213–216). Chi-square analysis detected significant linkage between four pairs of loci: ACO-1 and MNR-1, ACO-1 and PGM-1, PGM-1 and MNR-1, and MNR-1 and SKD-2. However, the linked loci used in the mating system analysis (ACO-1, MNR-1, and SKD-2) were not closely associated. The recombination fractions were 0.34 between ACO-1 and MNR-1 and between MNR-1 and SKD-2, while ACO-1 and SKD-2 and PGM-1 and SKD-2 assort independently. The linkage group, with recombination fractions, is as follows:



Linkage may affect the results of mating system analysis.

We used BIOSYS (Swofford and Selander 1981) to estimate percent polymorphic loci, alleles per locus, heterozygosity, Nei's (1978) genetic distance between populations, and Wright's (1965) F statistics, F_{IS} and F_{ST}, for the 1988 La Tinaja and 1997 El Butano samples. For small samples, BIOSYS calculates unbiased heterozygosity (Nei 1978). Fixation indices (F) within populations were calculated as the mean deviation of loci from Hardy–Weinberg proportions. Our inferences apply to the mature, cone-bearing trees in the populations.

The degree of genetic isolation between populations was estimated by Nm. Nm is the number of migrants per generation. Nm was calculated by two methods, i.e., by the relationship between F_{ST} and Nm and by the method of private alleles. From Wright (1951):

$$[1] \qquad Nm = \frac{1 - F_{ST}}{4F_{ST}}$$

Table 2. Allele frequencies for nine polymorphic loci in Martínez spruce.

	Population	
Locus and allele	La Tinaja	El Butano
ACO-1		
1	0.583	0.750
2	0.167	0.250
3	0.111	0.00
4	0.139	0.00
CAT-1		
1	0.917	1.000
2	0.083	0.00
CAT-2		
2	0.536	0.521
3	0.464	0.479
FEST		
1	1.000	0.958
2	0.00	0.042
GOT-3		
1	1.000	0.962
2	0.00	0.038
LAP-2		
1	0.781	0.875
2	0.219	0.125
MNR-1		
1	0.500	0.769
2	0.056	0.00
3	0.361	0.231
4	0.083	0.00
PGI-2		
1	1.000	0.885
2	0.00	0.115
SKD-2		
1	0.750	0.750
2	0.250	0.250

where F_{ST} is the proportion of the total genic diversity between populations. *Nm* can be calculated from the number and frequency of private alleles (unique alleles found in only one population) using simulations developed by Slatkin (1985):

$$[2] \qquad \log_{10}(\bar{p}(1)) = a \log_{10}(Nm) + b$$

where $\bar{p}(1)$ is the mean frequency of private alleles and *a* and *b* are constants determined by fitting simulated data. We used values for *a* and *b* developed for sample sizes of 10 and 25 (from Barton and Slatkin 1986). Because our mean sample size was 20.05, we corrected the estimates of *Nm* by factors of 10/20.05 and 25/20.05, respectively.

For mating system analysis, we used Ritland's (1986, 1989, 1990*a*, 1990*b*, 1994) MLTF and MLTR programs to calculate the single and multiple locus estimates of outcrossing rate, t_s and t_m, in all three samples. The Newton–Raphson method was used for iteration, and *p*, the allele frequency, and *t* were estimated jointly. Standard errors for t_m were taken from the estimated values in 200 bootstraps for the 1988 and 1994 samples and from 100 bootstraps for the larger 1997 sample. MLTR estimated the correlation of outcrossed paternity (*rp*) among progeny within a family for the 1997 sample from El Butano. Although adequate for estimation of population outcrossing rates, samples from La Tinaja and from El Butano in 1994 were too small to estimate family outcrossing rates; estimates failed to converge for most families. Outcrossing rates converged for all of the 26 families in the 1997 sample from El Butano, and chi-square contingency table analysis (Snedecor 1956, pp. 225–227) was used to test for differences among families. Expected values in the χ^2 test were derived from mean numbers of outcrossed and selfed progeny in the population.

Results

Of the 22 loci, 13 were monomorphic in both populations. Among the nine polymorphic loci (Table 2), eight alleles were unique (private alleles) to either La Tinaja or El Butano.

The unbiased estimates of expected heterozygosity were 0.101 at El Butano and 0.121 at La Tinaja, averaging 0.111 (Table 3). The means for percent polymorphic loci and the number of alleles per locus were about 32% and 1.39, respectively.

No locus deviated from Hardy–Weinberg expectations at La Tinaja, and observed heterozygosity was close to expected heterozygosity (Table 3). Two loci (LAP-2 and SKD-2) of nine deviated significantly from Hardy–Weinberg expectations at El Butano. Mean fixation index was near zero at La Tinaja, but positive at El Butano, reflecting a heterozygote deficiency.

Wright's *F* statistics (Table 4) indicated a very slight deficiency of heterozygotes overall (F_{IS} = 0.044). F_{ST} was 0.024, indicating that 97.6% of the genic diversity was within populations. Nei's genetic distance between La Tinaja and El Butano was only 0.003. *Nm*, the number of immigrants exchanged per generation, calculated from F_{ST}, was 10.17, a value that should preclude divergence due to random genetic drift. However, *Nm* estimated by the method of private alleles was 0.91 or 1.10 (mean of 1.01), based on Barton and Slatkin's (1986) simulations for samples of 10 or 25, respectively, and corrected for our mean sample size.

The results of the mating system analysis were most surprising (Table 5). The multilocus estimates of outcrossing rate (t_m) in the 1988 and 1994 collections was only 0.399 ($0.197 < t_m < 0.601$; $P = 0.95$) at La Tinaja and 0.685 ($0.465 < t_m < 0.905$; $P = 0.95$) at El Butano. Both estimates indicate a significant level of selfing. The estimate of the rate of selfing is highest for La Tinaja, the smallest population, although the confidence interval overlapped the estimate for El Butano. In both populations, the mean of single-locus estimates were slightly lower than the multilocus estimates (t_s was 0.358 for La Tinaja and 0.652 for El Butano), which may indicate some inbreeding from consanguineous mating other than selfing. However, the differences between the multilocus and the mean of single-locus estimates were not statistically significant.

Sample sizes for the 1994 collection from El Butano were especially small, so the mating system analysis was repeated using the 26 families and 996 progeny from the 1997 collection. Results were nearly the same; $t_m = 0.589$ ($0.475 < t_m < 0.703$; $P = 0.95$), indicating high levels of selfing. The multilocus estimate was almost identical to the mean of the single-locus estimates. Outcrossing rate varied significantly among families, from 0.00 to 1.00. The correlation of outcrossed paternity among progeny within a family was high, 0.389. Thus, the probability that two randomly chosen outcrossed progeny within a maternal family were full-sibs

Table 3. Genic diversity and fixation indices in Martínez spruce.

Population	n	H_e	H_o	P	A	F
La Tinaja	16.5	0.121 (0.047)	0.121 (0.047)	27.3	1.45 (0.19)	–0.058
El Butano	23.6	0.101 (0.035)	0.086 (0.031)	36.4	1.36 (0.10)	0.121
Mean		0.111	0.104	31.9	1.39	

Note: Standard errors are given in parentheses. The fixation index (F) is calculated as the mean for all polymorphic loci. n, mean sample size per locus; H_e, expected heterozygosity (unbiased estimate); H_o, observed heterozygosity; P, percent polymorphic loci; A, number of alleles per locus.

Table 4. Estimates of Wright's (1965) F statistics for nine polymorphic loci in Martínez spruce.

Locus	F_{IS}	F_{IT}	F_{ST}
ACO-1	–0.168	–0.129	0.033
CAT-1	–0.091	–0.043	0.043
CAT-2	0.038	0.039	0.000
FEST	–0.043	–0.021	0.021
GOT-3	–0.040	–0.020	0.020
LAP-2	–0.003	0.012	0.015
MNR-1	0.141	0.183	0.049
PGI-2	0.246	0.293	0.061
SKD-2	0.225	0.225	0.000
Mean	0.044	0.066	0.024

Table 5. Single locus and multilocus outcrossing estimates in two populations of Martínez spruce.

		El Butano	
Allozyme	La Tinaja	1994	1997
ACO-1[a]	0.666 (0.078)	0.095 (0.166)	—
GOT-3[a]	—	—	0.329 (0.107)
MNR-1[a]	0.229 (0.058)	0.701 (0.208)	0.586 (0.048)
PGI-2[a]	—	—	0.354 (0.052)
SKD-2[a]	0.671 (0.231)	0.705 (0.111)	0.738 (0.043)
Mean t_s[b]	0.358 (0.099)	0.652 (0.113)	0.589 (0.059)
t_m[b]	0.399 (0.101)	0.685 (0.110)	0.589 (0.057)

Note: Standard errors are given in parentheses.
[a] Calculated with Ritland's (1990*b*) MLTF.
[b] Calculated with Ritland's (1994) MLTR.

(i.e., had the same pollen parent) was 38.9%, which indicates a predominance of biparental crosses.

Discussion and conclusions

The population mean expected heterozygosity of 0.111 for Martínez spruce is decidedly less than that reported for most other spruces and for conifers in general (Hamrick and Godt 1989). In 30 studies of genic diversity in 10 species of spruce (reviewed in Ledig et al. 1997), heterozygosity generally ranged above 0.15. The only value lower than that estimated for Martínez spruce was 0.107 for black spruce (*Picea mariana* (Mill.) BSP) by Yeh et al. (1986), although other authors reported values for black spruce that ranged from 0.220 to 0.351. Percent polymorphic loci for Martínez spruce is lower than any of 22 estimates from 9 other species of spruce, which ranged from 37 to 92%. The number of alleles per locus is also lower in Martínez spruce; for 17 estimates in 8 species the range was 1.33 to 3.00 (reviewed in Ledig et al. 1997). Only Chihuahua spruce has comparable levels of genic diversity ($H_e = 0.093$, $P = 27\%$, $A = 1.37$; Ledig et al. 1997). The relatively low level of diversity in Martínez spruce is, perhaps, not surprising for a species that probably numbers a total of less than 800 trees, fragmented into widely separated populations.

Divergence between populations as measured by Nei's genetic distance (D) is no larger than that observed in other species of spruce with more continuous distributions (reviewed in Ledig et al. 1997). In Chihuahua spruce, mean D in a sample of 10 populations was an order of magnitude greater, 0.033, and even populations separated by only a few kilometres had values larger than that observed for Martínez spruce (Ledig et al. 1997).

Divergence between populations, measured by F_{ST}, was 2.4%, which is not high compared with 18 previous estimates of F_{ST} (or G_{ST}) for spruce species, which were as low as 0.9% (Ledig et al. 1997). However, G_{ST} in one investigation of Norway spruce was 12.0% (Muona et al. 1990), and in Tien-Shan spruce (*Picea schrenkiana* Fisch. et Mey.) it was 11.8% (Goncharenko et al. 1992). Furthermore, the variation between La Tinaja and El Butano is dwarfed by comparison with the 24.8% of the variation attributable to differences among populations in Chihuahua spruce (Ledig et al. 1997).

Nei's (1978) genetic distance can be used to put rough limits on the time (T) since isolation of the populations at La Tinaja and El Butano: $T = D/2\mu$, where μ is the mutation rate (Nei 1975). For assumed μ of 10^{-5} or 10^{-6} and D of 0.003, T would be only 150–1500 years. Nei (1975) suggested that the average rate of codon substitution detectable by isozyme electrophoresis was 10^{-7}, which results in an estimated time since divergence of 15 000 years. However, we feel that the higher rates of mutation are more reasonable, because evidence suggests that generational mutation rates in long-lived woody plants are higher (e.g., 10^{-3}) than those in other organisms (Klekowski and Godfrey 1989; Lowenfeld and Klekowski 1992). In any case, the calculation suggests contact during the current interglacial and implies recent fragmentation of the range.

Spruce is typically a genus of boreal and subalpine forest. However, spruce of undetermined species occurred as far south as the basin of México as recently as the interval 8000–7000 years BP (Lozano-García et al. 1993; M.d.S. Lozano-García, personal communication). The nearest occurrence of Martínez spruce is presently about 500 km north, and of Chihuahua spruce 730 km northwest, of México City, suggesting a retreat or retraction of ranges. The retreat was accompanied by fragmentation, and Mexican spruces now occur only in isolated pockets of favorable habitat.

Nm estimated from Wright's F_{ST} was within the range normally observed for spruces (Ledig et al. 1997), which might suggest that the two populations are in no danger of diverging because of drift. However, 10.17 cannot possibly reflect the current number of immigrants per generation because of the small size of the populations and their wide separation, over 100 km from the southern cluster to El Butano. *Nm* takes several hundred generations to reach equilibrium once gene flow has ceased (Slatkin and Barton 1989). A generation in Martínez spruce may mean 200 years or more; Müller-Using and Lässig (1986) reported ring counts on increment cores and estimated ages up to 304 years. If time since divergence is 1500 years or less and a generation is 200 years, perhaps less than 7 or 8 generations have elapsed since fragmentation. In the absence of ecological studies, however, the relation between longevity and "generations" is not clear. Nevertheless, the estimate of 10.17 immigrants per generation must certainly reflect past gene exchange and overestimate current levels of gene flow. On the other hand, one might argue that the estimate of 0.91 to 1.10 for *Nm* calculated from private alleles is biased by the small sample size. Many alleles could have been missed in the sample of 18 trees available for La Tinaja, however, five of the eight private alleles were found there.

Observed heterozygosity was close to expected heterozygosity at La Tinaja, although there was a significant heterozygote deficiency at two loci at El Butano. By contrast, the mating system analyses indicated outcrossing rates of only 39.9 and 58.9–68.5%, respectively, for La Tinaja and El Butano. In spruce, filled seed yield and germinability are substantially depressed after selfing (Franklin 1970). The lack of germinable seeds for 11 of 21 trees sampled at El Butano in 1994 might suggest high rates of selfing. However, pollen limitation and parthenocarpy cannot be ruled out in spruce.

The estimates of t_m indicate a high proportion of selfing. Because some linkage was detected among the loci used, these estimates deserve closer scrutiny. The multilocus mating system model assumes loci are independent; however, the single-locus model does not, of course, depend on the assumption of independent assortment. The estimates of t_m and t_s were very similar. Therefore, violation of the assumption has had little effect on the estimate of t_m reported here, perhaps, because linkage was weak.

Comparison of the inbreeding coefficients with estimates of the outcrossing rate present the paradox of a predominantly or moderately selfing conifer with little or no heterozygote deficiency. The equilibrium inbreeding coefficient, F_e, is related to the outcrossing rate, *t*, as (Allard et al. 1968)

$$[3] \qquad F_e = \frac{1-t}{1+t}$$

Substituting our estimates of t_m for *t* yields an estimated F_e of 0.430 for La Tinaja and 0.187 to 0.259, depending on sample year, for El Butano, compared with observed fixation indices of –0.058 and 0.121, respectively. Thus, the estimate of the equilibrium inbreeding coefficient is twice as high as the observed fixation index for El Butano and substantially higher than that observed for La Tinaja.

Obviously, forces other than the mating system, perhaps selection, must operate to keep the populations at La Tinaja and El Butano as close to Hardy–Weinberg equilibrium as they appear to be. Populations of mature conifers often have excess heterozygosity, while embryos have a heterozygote deficiency. For example, in Monterey pine (*Pinus radiata* D. Don) Plessas and Strauss (1986) demonstrated a heterozygote deficiency in embryos, a shift toward equilibrium expectations in saplings, and excess heterozygosity in young trees. This suggests that selection operates throughout the life cycle to remove homozygotes, which probably represent inbreds.

Ritland (1990*c*) proposed a method to estimate the inbreeding depression of selfed progeny from the inbreeding coefficient and the rate of selfing:

$$[4] \qquad w = 2\left(\frac{(1-s)F}{(1-F)s}\right)$$

where *w* is the fitness of selfs relative to outcrosses, *F* is the inbreeding coefficient estimated for the parental generation, and *s* is the rate of selfing in the progeny ($t = 1 - s$). The formula assumes that the population is in equilibrium, i.e., adult inbreeding coefficients and rates of selfing are constant from generation to generation. Although this is probably not the case, the model will tend to overestimate *w* and thus provide an upper bound to the fitness of selfs. For the 1997 sample from El Butano, which includes several families and large progeny arrays, $w = 0.395$, suggesting substantial selection against selfs between the seedling and the mature tree stage. The reliability of such estimates is likely to be poor but probably biased upwards (Ritland 1990*c*).

Most Pinaceae with appreciable levels of genic diversity suffer inbreeding depression when selfed because of a correlated genetic load (reviewed in Ledig 1986). Upon selfing, spruce have a 60% depression in filled seed yield, a 17% reduction in germination of filled seeds, a higher than normal frequency of mutant types, an age-dependent reduction in height growth of the germinants, and an 82% greater mortality, on average (Franklin 1970). Only Serbian spruce (*Picea omorika* (Pančić) Purk.) suffers no depression in seed yield from selfing (Langner 1959; Wright 1955), although selfed progeny are depressed in height at 2 years of age (Langner 1959).

No published studies of spruces have reported rates of selfing as high as those found here for Martínez spruce (i.e., 60% selfing at La Tinaja), except for the related Chihuahua spruce in which two small populations of 17 and 107 spruce had rates of selfing of 100 and 85%, respectively, based on small samples (Ledig et al. 1997). Norway spruce at the northern limits of its range in Finland (Muona et al. 1990) and white spruce (*Picea glauca* (Moench) Voss) in Newfoundland (Innes and Ringius 1990) have relatively low levels of outcrossing (74 and 73%, respectively). The low estimate for Norway spruce might reflect the fact that seeds were collected from lower branches, and selfing is more common in the lower crown. Other estimates of outcrossing rates in spruces range from 84 to 98% (Kuittinen and Savolainen 1992; Shea 1987; King et al. 1984; Chaisurisri et al. 1994; Cheliak et al. 1985). Even the rare Serbian spruce whose populations occupy, in total, only 60 ha in its native range and which is almost entirely self-fertile has an outcrossing rate of 84% (Kuittinen and Savolainen 1992).

However, a few conifers in the Cupressaceae have low rates of outcrossing; 32% in a western redcedar seed orchard (El-Kassaby et al. 1994) and as low as 57% in one natural population of northern white cedar (*Thuja occidentalis* L.; Perry and Knowles 1990). Some species of larch, which are in the Pinaceae, also may have low levels of outcrossing, varying from as low as 79 up to 91% in western larch (*Larix occidentalis* Nutt.; El-Kassaby and Jaquish 1996) and 54% in one population of eastern larch (*Larix laricina* (Du Roi) K. Koch) up to 71% in others (Knowles et al. 1987).

High rates of selfing in Martínez spruce may not be surprising. Neither the populations at La Tinaja or El Butano are large enough to produce an extensive pollen cloud (Jackson and Givens 1994), which suggests that mating would be predominantly by selfing or by pollination among nearest neighbors. The high correlation of outcrossed paternity within families of Martínez spruce suggests this. Within stands, trees also may differ in the date and length of anthesis, a common observation in other conifers (e.g., Eriksson et al. 1973; El-Kassaby et al. 1984; Griffin 1984), which would further restrict mating combinations.

On the other hand, such low rates of outcrossing never have been reported previously in spruces, except for the related Chihuahua spruce and therefore, are noteworthy. Martínez spruce illustrates the flexibility that is probably inherent in all conifer mating systems. Because self-incompatibility is unknown in spruce or pine, barriers to selfing are largely the result of inbreeding depression. Martínez spruce illustrates the capacity for facultative selfing, which may have facilitated the long persistence of conifers in the evolutionary landscape. It would also favor their ability to colonize new habitat, even to the point of being invasive (Bannister 1965).

While Martínez spruce provides no novel insights into how a conifer purges its load, it does provide a glimpse of the conditions under which the anomalous Pinaceae without genic diversity survived in refugia, purging their load through mixed selfing and outcrossing. Examples are red pine in glacial refugia during the Pleistocene and Torrey pine in interglacial refugia during the Holocene. Nevertheless, many such populations undoubtedly went extinct.

The infrequency of seed years and the failure of many trees to produce either cones or viable seeds threatens extinction of Martínez spruce. It is listed as “critically endangered” by the International Union for the Conservation of Nature (Farjon and Page 1999). The mating system analyses suggest that its low fecundity may be the result of inbreeding. Even in the mature trees that produced seeds during the course of this investigation, roughly half the progeny were from selfing events. This has drastic implications for conservation because of inbreeding depression.

Martínez spruce may become extinct as a result of catastrophe, such as fire, or from changing climate or from stochastic variation in conditions that affect seed production and seedling regeneration. Protection against fire and clandestine harvest should be strengthened. Even then, inbreeding depression may draw Martínez spruce into an extinction vortex (Gilpin and Soulé 1986). Controlled pollination could be used to produce outcrossed progeny for enrichment plantings, but this would be prohibitively expensive. To reduce natural selfing, gene flow within stands might be encouraged by thinning some of the intermixed pines and hardwoods. Enrichment plantings of spruce seedlings from collections, controlling their competition, and attempting to expand the limits of the spruce populations might be economically viable. For species like Martínez spruce, reduced to a last few fragments, ex situ conservation also is warranted. Seed banks or tissue cultures should be established in several locations, such as botanic gardens, as insurance against possible extinction in the wild (e.g., Ledig et al. 1998). If populations of Martínez spruce are lost in the wild, ex situ, collections could be used to restore the stands or create new populations in suitable habitat.

We do not know to what extent Martínez spruce has purged its genetic load of deleterious recessive alleles. The level of genic diversity is lower than that observed in many other species of spruce, perhaps suggesting that concurrent reduction in load has occurred. However, the high level of inbreeding depression, which is suggested by the estimated survival fitness of 0.395 relative to outcrosses at El Butano, indicates that considerable load remains. Whether Martínez spruce survives long enough to purge its load and tolerate selfing without reproductive loss, will in large part be determined by chance (Lande 1988) as well as genetic factors.

Acknowledgments

This study was an undertaking of the Forest Genetic Resources Study Group – North American Forestry Commission – Food and Agricultural Organization of the United Nations. It was completed with the help of National Research Initiatives Competitive Grant Program award No. 95-37101-1916 to the senior author. Some seed collections were funded by the USDA Office of International Cooperation and Development, project No. 190-6. We thank Sylvia R. Mori for her statistical review. We are grateful to many colleagues: Rufino Benitez-Trujillo, who made the seed collection from La Tinaja and attempted to collect from El Butano several times; David J. Wren for traveling to El Butano under (the usual) difficult circumstances; Carlos Ramirez-Herrera for the 1997 seed collection from El Butano; Burkhardt Müller-Using and Glafiro Alanís-Flores for sharing their information on Martínez spruce with us; Jaime E. Flores-Lara for his aid and hospitality; Teobaldo Eguiluz-Piedra for helping to initiate this project; Manuel Mápula-Larreta for his help in the field; Kermit Ritland for invaluable help in interpreting the mating system analyses; and two anonymous reviewers whose comments greatly improved the manuscript.

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