Evolution, 53(1), 1999, pp. 91–99

EVIDENCE FOR AN EXTREME BOTTLENECK IN A RARE MEXICAN PINYON: GENETIC DIVERSITY, DISEQUILIBRIUM, AND THE MATING SYSTEM IN *PINUS MAXIMARTINEZII*

F. THOMAS LEDIG,[1,2] M. THOMPSON CONKLE,[3] BASILIO BERMEJO-VELÁZQUEZ,[4] TEOBALDO EGUILUZ-PIEDRA,[4] PAUL D. HODGSKISS,[3] DAVID R. JOHNSON,[1] AND WILLIAM S. DVORAK[5]

[1]*Institute of Forest Genetics, Pacific Southwest Research Station, USDA Forest Service, 2480 Carson Road, Placerville, California 95667*

[2]*E-mail: tledig@ucdavis.edu*

[3]*Institute of Forest Genetics, Pacific Southwest Research Station, USDA Forest Service, Box 245, Berkeley, California 94701*

[4]*Centro de Genetica Forestal, Universidad Autonoma Chapingo, Apartado Postal No. 37, Chapingo, México, C.P. 56230, México*

[5]*CAMCORE, Grinnells Laboratories, College of Forest Resources, North Carolina State University, Box 7626, Raleigh, North Carolina 27695-7626*

Abstract.—Maxipinon (*Pinus maximartinezii* Rzedowski), which is confined to a single population of approximately 2000 to 2500 mature trees, covers about 400 ha in southern Zacatecas, Mexico. Genetic diversity measured by expected heterozygosity was 0.122, which is moderate for pines. However, percentage polymorphic loci was low, 30.3%. The fixation index (F) of 0.081 indicated only slight heterozygote deficiency. Mating system analysis indicated a significant but low level of selfing; the multilocus outcrossing rate, t_m, was 0.816. The mean of single locus estimates, t_s, was smaller (0.761), perhaps suggesting mating among relatives, although the difference between t_m and t_s was not statistically significant.

The most striking features of maxipiñon's genetic structure were that no polymorphic locus had more than two alleles and most alleles at polymorphic loci were at intermediate frequencies. This is in contrast to other pines, which often have three to five or more alleles at some loci and in which the distribution of allele frequencies is U-shaped, most alleles being present at frequencies less than 10% or greater than 90%. A population with only two alleles per locus and at intermediate frequencies could occur if the population had been reduced to an extreme bottleneck and then expanded rapidly before random drift modified allele frequencies. A novel origin from a hybridization event would also explain the results.

Significant gametic disequilibrium was detected at several pairs of loci in both maternal and paternal gametes. The presence of disequilibrium is in agreement with an origin from an extreme bottleneck, perhaps even a single seed. Furthermore, it demands that the event be relatively recent. The number of generations, as calculated from the observed mean disequilibrium, suggested that maxipiñon derived from an extreme bottleneck four to five generations ago, which is less than 1000 years in this species.

Key words.—Endangered species, isozymes, linkage disequilibrium, outcrossing, rarity.

Received December 11, 1997. Accepted September 30, 1998.

Maxipiñon (*Pinus maximartinezii* Rzedowski) provides an example of a species that may have survived an extreme bottleneck. This suggestion is based on allozyme frequencies, number of alleles per locus, and measures of disequilibrium. Alternatively, but less likely, the species could have originated as a single mutant individual or as a unique, monotypic hybrid between two other, unknown pines.

Maxipiñon is the strangest of the 12 to 15 known pinyon pines (*Pinus* subgenus *Strobus*, subsection *Cembroides* Engelm.). Most pinyon pines have small cones with fragile scales. The cone of maxipiñon is huge and woody, resembling the big-cone pines (subsection *Sabinianae* Loud.). However, the big-cone pines belong to the subgenus *Pinus* and the two subgenera, *Strobus* and *Pinus*, have been separated for at least 135 million years (Millar and Kinloch 1991; Millar 1993), so the relationship is only superficial. The relationship of maxipiñon to other pinyon pines is uncertain, but terpene analysis indicated a strong similarity to weeping piñon (*Pinus pinceana* Gordon; Zavarin and Snajberk 1987). Cladistic analysis of morphological data also indicated that these species were related, although both differed from Mexican piñon (*Pinus cembroides* Zucc.; Farjon and Styles 1997).

Maxipiñon is also one of the rarest of pines. It occurs in only a single location, between 1600 and 2250 m in elevation on a mesa called Cerro Piñones, which is the southern extreme of Sierra de Morones in the state of Zacatecas, Mexico. Its entire range was mapped as approximately 400 ha and the population estimated as 2000–2500 mature trees (Donahue and Mar-Lopez 1995). The calcareous soil and other site characteristics were described by Donahue and Mar-Lopez (1995).

Regeneration is sparse (Donahue and Mar-Lopez 1995) or practically nonexistent (Perry 1991) on Cerro Piñones. One reason is that the seeds, or "pine nuts," are prized for food and virtually the entire crop is harvested for consumption or for market. Indeed, the species was discovered in 1963 because Jerzy Rzedowski (1964) noted its unusually large seeds in the market at Juchipila, the nearest large town to Cerro Piñones. The seeds are among the largest in the pines, averaging about 900 per kilogram and selling for $4.00 to $8.00 (U.S. currency) per kilogram in the local market.

Removal of virtually the entire seed crop causes concern because of the potential impact on the ability of the species to sustain itself. Moreover, the seed-bearing cones are harvested destructively, by lopping off branches with a machete, and this may reduce the number of buds for future crops.

Other reasons for the lack of regeneration are the goats and cattle that graze beneath the trees. In addition, the tree

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has thin bark and is susceptible to fire (Donahue and Mar-Lopez 1995). One of us (TE-P) observed plentiful regeneration in 1985, but the stand was burned by wildfires in 1986 and 1989 (Emanoil 1994; Donahue and Mar-Lopez 1995), which perhaps explains the lack of regeneration found by Perry (1991) and Donahue and Mar-Lopez (1995).

By virtue of the population's restricted size and the lack of regeneration, maxipiñon is considered an endangered species (Emanoil 1994, Vera 1990). The entire population is on private land, making in situ conservation problematic. Central America and Mexico Coniferous Resource Cooperative (CAMCORE) has made seed collections for the purpose of ex situ conservation (Donahue and Mar-Lopwz 1995). These seeds have been planted in Brazil, South Africa, and Zimbabwe.

According to local farmers, much of the Sierra de Morones may have been covered with scattered maxipiñons as recently as 60 years ago. If disturbance is reducing the population in size, maxipiñon eventually may be condemned to increased inbreeding. Selfing is suggested by the occurrence of 77 albino mutants among 10,000 seedlings germinated for a conservation planting (T. Eguiluz-P., unpubl. data).

An analysis of variation in cone, seed, and needle morphology and needle anatomy was carried out by García-Nuñez and Eguiluz-Piedra (1985). They collected seeds and samples from 35 trees and analyzed 12 needle characters, 11 cone and scale dimensions, three seed traits, and two seedling traits. Highly significant differences were observed among trees in all but one trait (cone length:width ratio). However, the effects of environment and genotype were confounded in the cone, needle, and seed traits, and maternal effects could influence early seedling characteristics. Terpene analyses revealed only restricted variation among trees (Eguiluz-Piedra et al. 1985; Zavarin and Snajberk 1987). A great deal of genetic variation would be surprising considering the restricted range of maxipiñon.

Therefore, we analyzed allozyme variation to directly assess genetic diversity and genetic structure (i.e., gene and allele frequencies, level of heterozygosity, fixation index) in maxipiñon. We estimated the rate of outcrossing to determine whether inbreeding was a problem in the conservation of this species.

Materials and Methods

Isozymes of several seed collections were analyzed 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.

A random sample was taken from a 200 kg seedlot harvested for market in 1986. We will refer to this collection as "Market-1986." Eguiluz-Piedra et al. (1985) reported 900 seeds per kg, and each tree produces 1 to 2 kg of seeds; therefore a 200-kg lot was expected to represent more than 100 trees. In preparation for electrophoresis, seeds were scarified with pliers to crack the seed coat and then treated with 1% hydrogen peroxide for 48 hr in a refrigerator to stimulate metabolic activity (Leung and García 1985). The solution was changed after 24 hr. After treatment with hydrogen peroxide, seeds were imbibed on wet filter paper in petri dishes. Megagametophytes were dissected from the seeds, extracted, and analyzed for 26 presumptive loci after three days, about four days before germination usually takes place. For estimating allele frequencies, n varied from 90 to 130.

Results of the initial isozyme analysis seemed so novel that in 1990 we acquired a second collection, which represented an unknown number of trees from the 1986 cone crop, through Richard M. Jeffers of the USDA Forest Service's Southwestern Region. The collection consisted of only 100 seeds and the quality was poor. We will refer to this collection as "Jeffers-1986." Prior to processing, seeds were soaked for 24 hr in 0.1% hydrogen peroxide and then placed in petri dishes with 0.01% hydrogen peroxide for germination. When radicles emerged, megagametophyte and embryo pairs were dissected, separated, extracted, and analyzed for five polymorphic loci. Allele frequencies were calculated from the combined megagametophyte and pollen genotypes (maximum $n = 96 + 96 = 192$).

CAMCORE collected cones from the 1993 crop. After delays in clearing their export from Mexico, seeds from 50 trees became available for isozyme analysis in 1995. The 50 seed trees were distributed throughout the stand, and cones and seeds were maintained separate by tree. We will refer to this collection as "CAMCORE-1993." In June 1995, seeds were placed in petri dishes on moist sand for germination. When the radicle appeared through the seed coat, megagametophytes and embryos were dissected and separated for analysis. Unfortunately, seed viability proved very low (germination rate was 5.7%) and only 26 trees had any germinable seeds. Whether the problem was poor storage conditions or inherently short-lived seeds is not known. Megagametophyte-embryo pairs from 77 seeds were analyzed for five polymorphic loci. Because the number of seeds was insufficient to genotype the parent trees, only a limited analysis was possible. The megagametophyte genotype of the first seed from a tree was combined with data from all the pollen genotypes to calculate allele frequencies (maximum $n = 26 + 77 = 103$).

In 1995, the Centro de Genetica Forestal collected at least one cone from each of 100 trees distributed over a transect on Cerro Piñones that was roughly 550 m long and 150 m wide. We will refer to this collection as "CGF-1995." Seed viability was generally high: mean filled seed percentage was 81.8%, or 46.6 seeds, per cone. Limited germination tests resulted in 89% germination of filled seeds. Of the 100 trees, only four failed to provide adequate numbers (i.e., six seeds) for analysis. Allele frequencies were calculated from seed parent genotypes (maximum $n = 2 \times 96 = 192$).

Seeds from the 1995 collection were stored at 2°C until needed. In late 1996 and early 1997 they were prepared for isozyme analysis by imbibing them in a well-aerated water bath for 24 hr, followed by 12 days of cold stratification at 2°C. After stratification, the megagametophyte and embryo were excised and separated.

We report the results of the CGF-1995 collection in detail because it was the largest and because seeds were maintained separately by tree, which enabled us to evaluate the mating system of maxipiñon. Data from the Market-1986, Jeffers-1986, and CAMCORE-1993 collections are reported mainly

to verify the unique distributions of alleles and allele frequencies in maxipiñon.

Because the megagametophyte of pine 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 increased sample size. We assayed six megagametophytes per tree in CGF-1995. A sample of six 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 $2(1/2)^6 = 0.03$.

We simultaneously analyzed the embryo alongside its megagametophyte to characterize the mating system. Knowing the contribution of the egg (the haploid genotype of the megagametophyte) to the zygote, the pollen contribution can be deduced by subtraction, which makes it possible to detect some outcrossed embryos.

We chose 22 of the 96 trees for intensive mating system analysis and analyzed 14–16 additional megagametophyte-embryo pairs when sufficient seeds were available. The trees chosen were among the most homozygous. Our rationale for choosing relatively homozygous parents was that most polymorphic loci were at intermediate frequencies and, therefore, outcrosses could be detected with greater precision in homozygotes than in heterozygotes.

We used the techniques of starch gel electrophoresis described in the laboratory manual of Conkle et al. (1982) to assay 21 enzyme systems. The enzymes *Adh, Ala, Fest, Lap, Mnr, Pgi, Pgm*, and *Tpi* were run on a tris citrate/lithium borate buffer (buffer A of Conkle et al. 1982); *Gdh, Gly, Got, G6pd, Mpi*, and *Ugp* were run on a tris citrate/sodium borate buffer (buffer B of Conkle et al. 1982); and *Aco, Ald, Cadh, Idh, Mdh, 6Pg*, and *Skd* were run on a morpholine citrate buffer (buffer E of Conkle et al. 1982). 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, which has proven to be an almost invariably monomorphic species, were included on each gel to aid interpretation. Where several zones of activity were observed for a single enzyme, numerals following the enzyme abbreviation were used for identification. Thirty-three presumptive loci were consistently scored and used in the statistical analysis of CGF-1995. For the mating system analysis, we scored 10 polymorphic loci.

Data from the 22 trees used for mating system analysis were sufficient to test segregation ratios, using heterogeneity chi-square (Snedecor 1956). We used BIOSYS (Swofford and Selander 1981) to estimate genetic diversity and to test for deviations from Hardy-Weinberg equilibrium. Corrections were applied for small sample size. Because of our sampling procedures, our inferences apply to the population of mature, cone-bearing trees. We used Weir's (1990) program GAMETE.FOR to calculate disequilibrium for nine major polymorphic loci in the maxipiñon CGF-1995 collection. With nine loci, there are 36 two-locus combinations. We calculated disequilibrium for 576 maternal gametes (six from each of 96 trees) and 576 pollen gametes. We used Ritland's (1990) MLT program for mating system analysis and estimated standard errors of multilocus outcrossing rates (t_m) from 250 bootstraps for each tree. Because outcrossing rates greater than 1.00 are not biologically possible, we constrained estimates to an upper limit of 1.00, even though values of t_m greater than 1.00 are statistically possible.

TABLE 1. Allele frequencies at five polymorphic loci scored in each of four separate collections of maxipiñon.

		Collection-Crop Year							
		Market-1986		Jeffers-1986		CAMCORE-1993		CGF-1995	
Locus	Allele	f[1]	N[2]	f	N	f	N	f	N
Got3	1	0.26	130	0.29	192	0.17	103	0.24	192
	2	0.74		0.71		0.83		0.76	
Lap2	1	0.44	84	0.47	192	0.36	103	0.49	192
	2	0.56		0.53		0.64		0.51	
Pgi2	1	0.54	126	0.74	142	0.51	104	0.55	192
	2	0.46		0.26		0.49		0.45	
Mnr1	1	0.41	99	0.38	192	0.46	104	0.41	192
	2	0.59		0.62		0.54		0.59	
6Pg1	1	0.63	90	0.54	192	0.72	104	0.57	192
	2	0.37		0.46		0.28		0.43	

[1] Allele frequency.
[2] Number of genomes scored.

RESULTS

In general, allele frequencies for five polymorphic loci that were scored in all of the four seed collections were in fairly good agreement among collections (Table 1). Chi-square indicated significant differences among collections in allele frequencies at *Pgi2* as a result of deviations in Jeffers-1986, and at *6Pg1* as a result of deviations in CAMCORE-1993. Jeffers-1986 and CAMCORE-1993 were the two poorest collections because they sampled the fewest maternal trees and because of their low seed viability. The two most extensive collections, Market-1986 and CGF-1995, did not differ significantly in allele frequencies.

Of the 33 loci scored in the CGF-1995 collection, 10 (30.3%) were polymorphic (Table 2). Significant segregation distortion was observed only at *6Pg1*, even though the pooled data were 81:77, close to expectations. The reason was significant heterogeneity among trees; two of eight trees that were tested for segregation at *6Pg1* deviated from the expected 1:1 ratio. *Aco1, Ala, Ald1, Adh, Cadh, Fest, Gdh, Got1, Got2, Gly, G6pd, Idh1, Idh2, Lap1, Mdh1, Mdh2, Mdh3, Mdh4, Pgi1, 6Pg2, Skd2, Tpi2*, and *Ugp* were all monomorphic. All polymorphic loci except *Skd1* were in Hardy-Weinberg equilibrium. Genotype frequencies at *Skd1* deviated from expectations ($P = 0.024$) because of a slight excess of homozygotes.

Expected heterozygosity in the CGF-1995 trees was 0.122 (± 0.036 standard error) and observed heterozygosity was 0.110, which suggests a deficiency of heterozygotes, although the expected and observed heterozygosities were within a standard error of each other. Fixation indices (F) varied

TABLE 2. Allele frequencies (f) and fixation indices (F) for 10 polymorphic loci in maxipiñon in CGF-1995.

	f			
	Allele 1	Allele 2	n^1	F
Ald2	0.615	0.385	96	0.033
Got3	0.240	0.760	96	0.085
Lap2	0.490	0.510	96	0.125
Mnr1	0.589	0.411	96	0.158
Mpi	0.761	0.239	71	−0.005
Pgi2	0.552	0.448	96	0.130
Pgm	0.995	0.005	96	0.225
6Pg1	0.568	0.432	96	0.032
Skd1	0.786	0.214	96	−0.083
Tpi1	0.561	0.439	82	0.109
Mean				0.081

[1] Number of diploid individuals sampled.

among the 10 polymorphic loci (Table 2), but were generally positive (eight of 10 loci), and averaged 0.081. *Pgm* may have contributed unduly to the mean. We observed an allelic variant at the *Pgm* locus in only one tree. With *Pgm* excluded, F was 0.065.

In each of the four seed collections, the polymorphic loci were notable for two reasons: there were never more than two alleles at a locus and allele frequencies were generally intermediate with the exception of *Pgm*. Altogether we sampled 192 genomes (2 × 96) from the seed parents and 1007 pollen parent genomes from embryos in CGF-1995, for a total of 1199 genomes, and never found more than two alleles at a locus. A sample of 1199 should be capable of detecting an allele with a frequency of 0.001 in a population of 2500 trees (Sjögren and Wyöni 1994), and the probability of detecting rare alleles is increased when considering 33 loci. We never observed a third allele in Market-1986, Jeffers-1986, or CAMCORE-1993. Therefore, with a high degree of certainty, maxipiñon has only two alleles per locus.

At nine of 10 polymorphic loci, allele frequencies were nearly intermediate. At the *Pgm* locus, a single heterozygote was observed among the 96 seed parents in CGF-1995, and allele frequencies were 0.005:0.995. Three of the other nine polymorphic loci deviated from truly intermediate frequencies (i.e., 0.50:0.50), and the greatest deviation was a 0.79:0:21 ratio at *Skd1*.

Significant disequilibrium ($P \leq 0.05$) was found for eight combinations in the maternal gametes and 10 in the pollen gametes (Table 2). This is more than would be expected by chance. For the 13 combinations that were in significant disequilibrium in either the pollen or maternal datasets, the same sign was found in 11 cases. That is, if the disequilibrium indicated coupling phase in the pollen, it indicated coupling in the maternal gametes, or if it indicated repulsion phase in the pollen, it also indicated repulsion in the maternal gametes for 11 of the 13 two-locus combinations. The probability of this occurring 11 or more times in 13 combinations if, in fact, the null hypothesis was true, is only 0.011. For the total set of 36 combinations, both maternal and pollen disequilibria have the same sign significantly (chi-square, $P < 0.01$) more often than expected by chance.

The mating system analysis in CGF-1995 showed maxipiñon to be largely outcrossing, but the multilocus outcrossing rate, t_m, was significantly less than 1.00. For the 22 trees with large numbers of gametophyte-embryo pairs (average of 19.6 pairs per maternal parent), t_m was 0.816 ($0.742 < t_m < 0.912$; $P = 0.95$). The mean of single locus estimates, t_s, was lower, 0.761, but the difference was not statistically significant.

TABLE 3. Multilocus outcrossing rate (t_m) based on 10 polymorphic loci in 22 families of maxipiñon in CGF-1995.

Family	n^1	t_m (SE)
6	21	0.97 (0.04)
22	20	0.42 (0.13)
28	22	0.46 (0.11)
30	20	0.83 (0.11)
31	22	0.42 (0.11)
32	15	1.00 (0.00)
41	20	0.86 (0.08)
42	20	0.45 (0.10)
44	22	0.96 (0.05)
47	20	0.96 (0.06)
50	22	0.96 (0.04)
55	20	0.87 (0.08)
59	14	0.94 (0.06)
60	21	0.73 (0.10)
66	21	1.00 (0.00)
77	20	0.86 (0.07)
79	22	0.92 (0.06)
82	20	0.76 (0.13)
85	20	1.00 (0.04)
89	20	1.00 (0.00)
94	9	1.00 (0.00)
96	20	0.82 (0.09)

[1] Number of embryos analyzed.

Considerable variation in outcrossing rate occurred among the 22 trees (Table 3). Estimates of t_m ranged from 0.42 to 1.00 and the distribution was bimodal; estimates of t_m were between 0.42 and 0.46 for four trees and between 0.92 and 1.00 for 11, and standard errors were 0.13 or less. Eighteen of the 22 trees had estimates of t_m greater than 0.73, and 16 of these had outcrossing rates that did not differ significantly from 1.00, that is, complete outcrossing. The differences among trees in outcrossing rate were statistically significant by chi-square test.

Outcrossing rates were also calculated from the six gametophyte-embryo pairs in each of the other 74 seed trees from CGF-1995. The multilocus estimate of outcrossing was 0.91, which is within two standard errors of the estimate from the sample of 22 relatively homozygous trees ($t_m \simeq 0.082$). As in the more intensive sample from the 22 families, the single-locus estimate was lower than the multilocus estimate, 0.86 in this case.

DISCUSSION

Maxipiñon is not genetically depauperate, but the percent polymorphic loci (30.3%) was much lower than that observed in most pines and spruces. Percent polymorphic loci within pine and spruce populations averaged 65.6% over 91 studies that included 42 species of pines (Ledig 1998) and 62.5% over 22 studies that included nine species of spruces (Ledig et al. 1997). Only nine of 111 estimates of the proportion of polymorphic loci in 49 species of pines were lower than the estimate for maxipiñon, and seven of these estimates were

from three species with either no genetic diversity or very low levels of variation: Aleppo pine (*P. halepensis* Mill.), red pine (*P. resinosa* Ait.), and Torrey pine (*P. torreyana* Parry). The other two estimates were from species with fragmented ranges: Swiss stone pine (*P. cembra* L.) and bishop pine (*P. muricata* D. Don). Furthermore, many studies reported in the literature considered fewer loci than reported here, and percent polymorphic loci is a statistic inversely dependent on the number of loci assayed.

Despite the low percent polymorphic loci, expected heterozygosity in maxipiñon was nearly equal to that in most other pine species (Ledig 1998) because expected heterozygosity is maximized when alleles are in intermediate frequencies, as in this case. Genetic diversity in maxipiñon can be compared to that in weeping piñon, which may be one of its nearest relatives. At a common set of 27 loci, expected heterozygosity was 0.127 in maxipiñon and 0.128 to 0.187 in weeping piñon, depending on population (F. T. Ledig, P. D. Hodgskiss, H. Sbay, and M. A. Capó-Arteaga, unpubl. data). From their review of the literature on pines, Hamrick and Godt (1996) reported an average expected heterozygosity of 0.157. Percent polymorphic loci in weeping piñon ranged from 44.4% to 74.1% compared to 29.6% for the same set of 27 loci in maxipiñon.

The number of alleles per polymorphic locus was two, which is highly unusual in conifers. Polymorphic loci commonly have multiallelic systems. For example, 17 of 21 loci in pitch pine (*P. rigida* Mill.), which has an expected heterozygosity only slightly greater than that of maxipiñon, had more than two alleles, ranging from three to five (Guries and Ledig 1982). Weeping piñon averages 3.0 to 3.5 alleles per polymorphic locus, depending on population, and some loci have five or six alleles (F. T. Ledig, P. D. Hodgskiss, H, Sbay, and M. A. Capó-Arteaga, unpubl. data). The presence of only two alleles at a locus suggests that maxipiñon may have survived an extreme bottleneck, perhaps even tracing its ancestry to a single seed in the recent past, because a single seed in a diploid can carry no more than two alleles at a locus. Apparently, the time since the bottleneck has been insufficient to generate many new variants by mutation; only one rare allele was found, a single heterozygote at *Pgm*, an otherwise monomorphic locus. Betancourt et al. (1991) used a similar line of reasoning when they argued that the presence of three or more alleles at a locus in pinyon pine (*P. edulis* Engelm.) indicated that the isolated population they studied was founded by more than one seed.

The distribution of allele frequencies in maxipiñon tends to confirm an extreme bottleneck followed by a rapid expansion that mitigated the effects of random genetic drift (see below for a model of effective population number during expansion following a bottleneck). Allele frequencies in almost all natural populations have a U-shaped distribution. That is, most alleles are either very common or rare. In 138 species or subspecies of animals, all allele frequency distributions were U-shaped, and there was never in any of the 138 species an indication of a mode at the intermediate gene frequency (Chakraborty et al. 1980). In maxipiñon, by contrast, allele frequencies were close to 0.50:0.50 at five of 10 polymorphic loci, and at four others, the maximum deviation was 0.786:0.214. Compared to pitch pine, another species

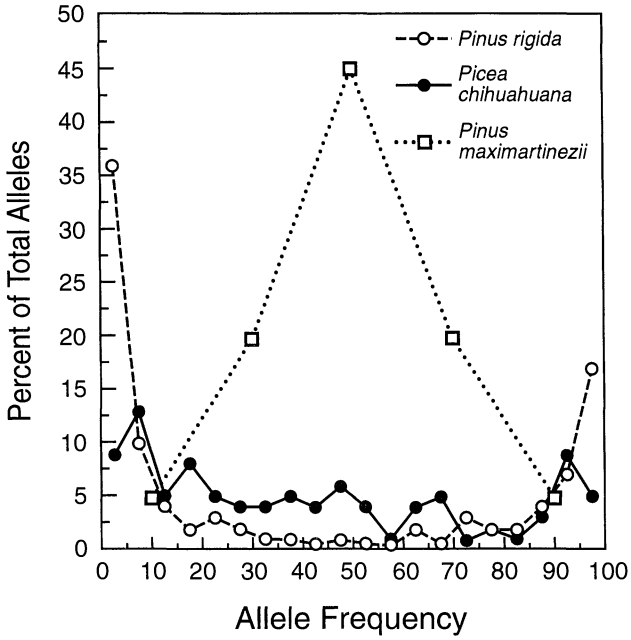

FIG. 1. Frequency distribution of allele classes in eight populations of pitch pine (*Pinus rigida*), 10 populations of Chihuahua spruce (*Picea chihuahuana*), and maxipiñon (*Pinus maximartinezii*). Allele frequencies in pitch pine have a typical U-shaped distribution with many alleles in frequencies less than 0.10 or greater than 0.90; Chihuahua spruce has a relative paucity of rare alleles as a result of random drift and fixation in small populations; in maxipiñon most alleles are in intermediate frequencies. Note, because maxipiñon has only one population and relatively few polymorphic loci, class intervals are 0.20 to accentuate the frequency distribution; the class interval for the other two species is 0.05.

that we have surveyed (Guries and Ledig 1982), rare alleles (i.e., alleles that were present, but in frequencies less than 10%) and their complement, the most common allele at a polymorphic locus (i.e., alleles with frequencies greater than 90% but less than 100%) accounted for 325 of 459 cases (i.e., 70% of the polymorphic loci). In maxipiñon only one rare variant was observed in the 27 to 33 loci surveyed in the combined sample (sampling with replacement) of over 1400 haploid genomes from the four separate collections. Figure 1 illustrates the distribution of allele frequencies in three species: maxipiñon; pitch pine, which is a wide-ranging conifer; and Chihuahua spruce (*Picea chihuahuana* Martínez), a species in which populations are small and isolated with minimal migration, so that gene frequencies seem to be determined largely by drift. If a single seed gave rise to the present population of maxipiñon, initial gene frequencies would of necessity be 0.50:0.50 in the absence of drift.

Linkage disequilibrium is expected if maxipiñon suffered an extreme bottleneck relatively recently. Physical linkage is not necessary to detect linkage disequilibrium. Loci that enter a cross together (as in a single seed) are associated for several generations even if they assort independently and are in Hardy-Weinberg equilibrium when considered separately. Of course, the greater the actual linkage between the loci, the longer the association lasts. Disequilibrium between two loci is indicated by a preponderance of gametes in the coupling phase (e.g., A_1B_1 and A_2B_2) over those in the repulsion phase

TABLE 4. Linkage disequilibrium (Δ) for two-locus pairs, their chi-square values (χ^2), and the number of generations (g) since disequilibrium was established, calculated under assumptions described in the text, in maternal and paternal gametes of maxipiñon in CGF-1995. Number of gametes varied from 488 to 576.

	Maternal gametes			Pollen gametes		
Loci	Δ	χ^2	g	Δ	χ^2	g
Ald2, Got3	0.0153	2.897	4.03	−0.0009	0.010	8.12
Ald2, Lap2	−0.0188	3.197	3.73	−0.0210	4.211*	3.57
Ald2, Pgi2	−0.0059	0.319	5.41	−0.0090	0.798	4.80
Ald2, 6Pg1	−0.0145	1.971	4.10	−0.0137	1.781	4.19
Ald2, Skd1	−0.0025	0.079	6.67	−0.0143	2.523	4.13
Ald2, Mnr1	0.0068	0.408	5.20	0.0204	4.164*	3.62
Ald2, Mpi	0.0083	0.742	4.91	0.0172	3.077	3.86
Ald2, Tpi1	−0.0074	0.503	5.08	0.0044	0.185	5.83
Got3, Lap2	−0.0062	0.482	5.34	−0.0164	3.179	3.93
Got3, Pgi2	0.0169	3.673	3.88	0.0201	4.902*	3.63
Got3, 6Pg1	−0.0219	5.997*	3.51	0.0140	2.195	4.16
Got3, Skd1	−0.0290	14.826*	3.11	−0.0092	1.242	4.76
Got3, Mnr1	0.0295	10.969*	3.08	0.0268	9.036*	3.22
Got3, Mpi	0.0070	0.736	5.16	−0.0079	0.811	4.98
Got3, Tpi1	−0.0149	2.834	4.07	−0.0012	0.016	7.73
Lap2, Pgi2	−0.0348	11.423*	2.84	−0.0337	10.519*	2.89
Lap2, 6Pg1	−0.0107	1.035	4.55	−0.0125	1.342	4.33
Lap2, Skd1	−0.0081	0.845	4.95	0.0043	0.213	5.84
Lap2, Mnr1	0.0133	1.624	4.23	0.0020	0.037	6.99
Lap2, Mpi	−0.0168	3.083	3.90	0.0018	0.033	7.09
Lap2, Tpi1	−0.0117	1.277	4.42	−0.0118	1.252	4.41
Pgi2, 6Pg1	0.0246	5.581*	3.35	0.0135	1.619	4.22
Pgi2, Skd1	−0.0242	7.646*	3.37	−0.0285	9.470*	3.13
Pgi2, Mnr1	0.0130	1.587	4.26	0.0158	2.474	3.98
Pgi2, Mpi	0.0114	1.465	4.45	−0.0129	1.690	4.28
Pgi2, Tpi1	−0.0016	0.024	7.31	−0.0117	1.272	4.42
6Pg1, Skd1	0.0131	2.244	4.26	0.0157	2.784	3.99
6Pg1, Mnr1	−0.0173	2.672	3.86	−0.0146	1.962	4.10
6Pgi, Mpi	0.0087	0.827	4.84	−0.0008	0.006	8.35
6Pgi, Tpi1	−0.0431	17.181*	2.54	−0.0455	17.945*	2.46
Skd1, Mnr1	−0.0222	6.330*	3.49	−0.0177	3.862*	3.82
Skd1, Mpi	−0.0122	2.291	4.36	0.0200	4.978*	3.64
Skd1, Tpi1	−0.0049	0.312	5.68	−0.0006	0.004	8.64
Mnr1, Mpi	−0.0150	2.441	4.06	−0.0019	0.040	7.00
Mnr1, Tpi1	0.0071	0.473	5.13	0.0205	4.081*	3.61
Mpi, Tpi1	0.0025	0.069	6.66	0.0033	0.111	6.23
Mean			4.44			4.83

* Probability of a chi-square this large or larger by chance is less than 0.05.

(A_1B_2 and A_2B_1), or vice versa. Several of the two-locus combinations in maxipiñon were in disequilibrium and the direction of disequilibrium was generally the same in maternal and paternal gametes. Linkage disequilibrium has rarely been detected in conifers, and never in such high frequency (Muona 1982).

Once established, disequilibrium dissipates rapidly in succeeding generations, most rapidly if the loci reside in different linkage groups. The disequilibrium (Δ) remaining after g generations as a fraction of the original disequilibrium (Δ_o) is $\Delta/\Delta_o = (1 - c)^g$, where c is the recombination fraction between the loci.

In the present case, we assumed that loci were not linked and, therefore, c was 0.5. The assumption is probably valid because we could find no evidence from previous studies in our laboratory that maxipiñon's 10 polymorphic loci were linked in other pines, and linkage relationships in pines are highly conserved (Conkle 1981). Loci detected here (e.g., *Got1, Got2, Got3, Lap1, Lap2*) correspond to the loci we have identified in other pines (Conkle 1981, unpubl. data), but comparisons among laboratories are probably not valid. For six combinations of loci, we were able to test for linkage. We detected significant linkage between *Ald2* and *Got3*. In four trees, the estimated recombination fraction was 0.20, 0.32, 0.35, and 0.45, respectively, with a mean of 0.33. Linkage this weak is close enough to independent assortment to ignore for the purposes of our analysis.

If maxipiñon descended from a single seed, initial disequilibrium (Δ_o) was 0.25. That is:

$$\Delta_o = \begin{vmatrix} 0.5 & 0 \\ 0 & 0.5 \end{vmatrix} = 0.25,$$

for coupling phase or −0.25 for repulsion phase.

Under these assumptions, we solved for the number of generations, g, since disequilibrium was established. The range in estimates was relatively small; g varied from 2.54 to 9.98 in the maternal gametes and from 2.46 to 8.64 in the pollen gametes (Table 4). The means and confidence intervals ($P = 0.95$) were $4.08 < 4.44 < 4.79$ for the maternal gametes

and $4.28 < 4.83 < 5.38$ for the pollen gametes. The two estimates overlap and suggest that the present population of maxipiñon went through a bottleneck about four to five generations ago. Note that because g varies approximately as the logarithm of Δ, it is relatively insensitive to variation in Δ. Doubling Δ would change g by a factor of about 0.67 to 0.90 in the region of interest.

Passini (1985) aged old trees in the maxipiñon population and found that they were 200 to 220 years old. Two hundred years is not necessarily the generation length, and generations likely overlap. Nevertheless, in tree species, stand regeneration usually follows major events like wildfire even though seeds may be produced every year. Given an apparent maximum age of about 200 years and a bottleneck four to five generations ago, the bottleneck must have been at least as recent as 800 to 1000 years ago.

The fixation index, 0.081, indicates only a moderate deficiency of heterozygotes despite the closed nature of the population and the smaller populations that must have existed in the last 1000 years if maxipiñon went through an extreme bottleneck. The observed heterozygote deficiency was in agreement with an estimated multilocus outcrossing rate of about 0.82. In a closed population at equilibrium, the fixation coefficient (F_e) is related to the outcrossing rate (t) as $F_e = (1 - t)/(1 + t)$ (Allard et al. 1968). Using the observed outcrossing rate of 0.816, F_e is calculated as 0.101, which is close to the estimated F of 0.081.

Although not significant, the single-locus estimate of outcrossing was lower than the multilocus estimate, which suggests inbreeding that results from crossing among related individuals. Crosses between relatives is not unlikely in maxipiñon because dispersal of its heavy, wingless seeds may be entirely by gravity, resulting in clustering of siblings. We realize that most pinyons and other wingless-seeded pines are bird-dispersed, but know of no avian seed dispersion in maxipiñon.

The presence of a rare allele at the *Pgm* locus provided an opportunity to measure gene movement by pollen within the area sampled. Only four embryos from three trees (excluding the putative selfs) among the total 853 analyzed received the rare allele, which meets the expected frequency of 4.4 based on 96 pollen donors, each with two alleles. All of the trees receiving the rare *Pgm* allele were within approximately 65 m of the putative donor. Of course, there may be other carriers of *Pgm2* that were missed in our sample.

Maxipiñon was predominantly outcrossing on the population level, but among individual trees the distribution of t_m was bimodal. A wide range in outcrossing among trees is not usually observed in highly outcrossing pines, but is not uncommon in woody species with a mixed mating system. For example, the range was 0.25 to 1.00 in northern white-cedar (*Thuja occidentalis* L.), 0.00 to 1.00 in western red-cedar (*Thuja plicata* Donn ex D. Don), and approximately 0.4 to 1.0 in western larch (*Larix occidentalis* Nutt.), all of which are conifers (Perry and Knowles 1990; El-Kassaby et al. 1994; El-Kassaby and Jaquish 1996). In tropical angiosperms with mixed mating systems, t_m often ranges from 0.40 to 1.00 and even from 0.00 to 1.00 (e.g., Murawski et al. 1990; Murawski and Hamrick 1992; Murawski et al. 1994; Boshier et al. 1995).

TABLE 5. Mean number of filled seeds per cone and filled seeds as a percentage of total seeds per cone averaged by outcrossing class in maxipiñon.

Outcrossing Class	n[1]	Filled Seed	
		Number	% of total
42–46%	4	49.8	90.3
70–89%	7	51.7	81.3
92–100%	11	40.4	72.6

[1] Sample size; that is, number of trees in the outcrossing class.

We examined seed yields in maxipiñon to see if they were related to variation in the rate of outcrossing (Table 5). Four of the 22 trees in the intensive mating system analysis had outcrossing rates equal to or less than 0.46 (mean of 0.44) and 11 had rates equal to or greater than 0.92 (mean of 0.97). The remaining seven trees had outcrossing rates ranging from 0.73 to 0.87 with a mean of 0.82. The trees with the highest rates of selfing (lowest rate of outcrossing) were no more isolated than those with low rates of selfing.

However, the trees with the highest rate of selfing had more filled seeds per cone (49.8 vs. 40.4) and a higher percentage of filled seeds (90.3% vs. 72.6%) than the trees with the highest rate of outcrossing. The difference was significant ($P = 0.05$) for filled seed percentage. Trees in the intermediate category for outcrossing rate were intermediate for percent filled seed and only slightly higher than the trees in the low outcrossing category in seed yield per cone. The higher values for filled seed percentage in the trees with high rates of selfing may indicate that they carry few or no deleterious alleles (genetic load), so selfing results in little loss of reproductive output. Conversely, high outcrossing rates may be an artifact of embryo abortion resulting from homozygosis for recessive lethal equivalents.

Inbreeding does not seem to be a major problem for the conservation of maxipiñon because filled seed percentages were high; they averaged 81.8% for the 100 trees in the CGF-1995 collection. Inbreeding depression in pines is usually first expressed as reduced seed set. It would be interesting to artificially self-pollinate trees of maxipiñon. We predict that little reduction in seed set would be expected relative to the average reduction of 33% following selfing of pines in general (Franklin 1970).

Drift and fixation of alleles has been invoked to explain patterns of variation in conifers such as red pine and Torrey pine (Fowler and Morris 1977; Ledig and Conkle 1983). However, in those cases, populations were without genetic variation, which suggests that they were restricted to very small numbers for several generations. Based on the calculated time since disequilibrium was established in maxipiñon (four to five generations) and the present size of the population, it must have expanded rapidly.

Like most conifers, maxipiñon, is potentially capable of rapid expansion from a bottleneck. Mature trees at Cerro Piñones produce about 1–2 kg of seeds, about 900–1800 seeds, per year (Eguiluz-Piedra et al. 1985). If only 0.05% of the seeds that were produced over, for example, 100 years of reproductive maturity survived, the exponential increase would easily account for the present population in four generations. If the original parent produced 50 surviving prog-

eny, an exponential increase of 1.26 would result in a population of 2496 after four generations. Under this model, the population numbers would be 50, 138, 497, and 2496, and the effective population number, N_e, for the four generations would be about 135. An N_e of 135 is large enough to keep the effects of drift to minimal levels.

A valuable next step would be to identify the progenitor(s) of maxipiñon. Any candidate must be variable at the polymorphic loci (excluding *Pgm*) identified here. However, the possibility also exists that maxipiñon combines the genomes of two other taxa, which must in sum be polymorphic at the nine major loci. Weeping piñon or Nelson piñon (*Pinus nelsonii* Shaw) are prime candidates. Based on the estimated disequilibria, the likely genomes that contributed to maxipiñon were: (1) *Ald2-1, Got3-1, Lap2-2, Pgi2-1, 6Pg1-1, Skd1-2, Mnr1-1, Mpi-2, Tpi1-2*; and (2) its converse, *Ald2-2, Got3-2, Lap2-1, Pgi2-2, 6Pg1-2, Skd1-1, Mnr1-2, Mpi-1, Tpi1-1*, where the hyphenated numerals designate an allele at a locus.

The possibility cannot be discounted that maxipiñon is a recent species. It is not difficult to imagine that agriculturists capable of breeding maize from a weedy grass in a few thousand years could opportunistically select and propagate a large-seeded piñon. The Mayans apparently conserved and even introduced useful tree species into their walled selvas in the Yucatan, and elsewhere in Mexico, cherry (*Prunus serotina* ssp. *capuli* Cav.), crabapple (*Crataegus mexicana*), and leucaena (*Leucaena* spp. Benth.) were selectively promoted (Gomez-Pompa et al. 1987; Bye 1993).

The universality of biallelic isozymes, the clustering of alleles at intermediate gene frequencies, and the presence of gametic disequilibrium considered together indicate an extreme bottleneck. Although each considered independently might be unconvincing, together they suggest that the present population possibly traces its origin to a single seed. Whatever the nature or size of the bottleneck, its causes should be sought within the last 1000 years, and most probably between 400 and 1000 years ago.

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. Research was partially funded by the USDA Office of International Cooperation and Development through project grant no. 190-6 and by a technical exchange grant in 1992. We thank M. Mápula-Larreta for organizing the 1995 cone collection of maxipiñon, J. L. Campo-Diaz for introducing us to Cerro Piñones, and R. M. Jeffers for providing seeds. We are especially grateful to R. D. Westfall for his help in mating system analysis. We acknowledge, with thanks, the helpful reviews by S. R. Mori, D. Piñero, M. M. Sewell, and two anonymous reviewers.

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Corresponding Editor: D. Piñero