Molecular Ecology (2006) **15**, 2787–2800 doi: 10.1111/j.1365-294X.2006.02974.x

Decoupled mitochondrial and chloroplast DNA population structure reveals Holocene collapse and population isolation in a threatened Mexican-endemic conifer

JUAN P. JARAMILLO-CORREA,* JEAN BEAULIEU,*† F. THOMAS LEDIG‡ and JEAN BOUSQUET*
Chaire de recherche du Canada en génomique forestière et environnementale and Centre de recherche en biologie forestière, Pavillon Charles-Eugène-Marchand, Université Laval, Québec, Canada G1K 7P4, †Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, 1055 Rue Du P.E.P.S., PO Box 10380 Sainte-Foy, Québec Str., Québec, Canada G1V 4C7, ‡Institute of Forest Genetics, Pacific Southwest Research Station, USDA Forest Service, and Department of Plant Science, University of California, Mail Stop 6, 1 Shields Avenue, Davis, California 95616, USA

Abstract

Chihuahua spruce (*Picea chihuahuana* Martínez) is a montane subtropical conifer endemic to the Sierra Madre Occidental in northwestern México. Range-wide variation was investigated using maternally inherited mitochondrial (mtDNA) and paternally inherited chloroplast (cpDNA) DNA markers. Among the 16 mtDNA regions analysed, only two mitotypes were detected, while the study of six cpDNA microsatellite markers revealed eight different chlorotypes. The average cpDNA diversity (H = 0.415) was low but much higher than that for mtDNA (H = 0). The distribution of mitotypes revealed two clear nonoverlapping areas ($G_{ST} = N_{ST} = 1$), one including northern populations and the second one including the southern and central stands, suggesting that these two regions may represent different ancestral populations. The cpDNA markers showed lower population differentiation (G_{ST} = 0.362; R_{ST} = 0.230), implying that the two ancestral populations continued to exchange pollen after their initial geographic separation. A lack of a phylogeographic structure was revealed by different spatial analyses of cpDNA ($G_{ST} > R_{ST}$; and SAMOVA), and reduced cpDNA gene flow was noted among populations (Nm = 0.873). Some stands deviated significantly from the mutation–drift equilibrium, suggesting recent bottlenecks. Altogether, these various trends are consistent with the hypothesis of a population collapse during the Holocene warming and suggest that most of the modern *P. chihuahuana* populations are now effectively isolated with their genetic diversity essentially modelled by genetic drift. The conservation efforts should focus on most southern populations and on the northern and central stands exhibiting high levels of genetic diversity. Additional mtDNA sequence analysis confirmed that *P. martinezii* (Patterson) is not conspecific with *P. chihuahuana*, and thus deserves separate conservation efforts.

Keywords: chloroplast DNA, conservation genetics, México, mitochondrial DNA, *Picea chihuahuana*, *Picea martinezii*, postglacial history

Received 22 November 2005; revision accepted 17 March 2006

Introduction

The study of the postglacial history of forest trees in North America has been generally limited to boreal and temperate species whose modern ranges were partially or totally covered by glacial ice sheets (e.g. Davis 1983; Jackson *et al*. 1997). Generally, it has been inferred that during the last glacial maximum, these species were confined to scattered and isolated glacial populations or refugia. During the ensuing Holocene warming, most of these populations expanded and migrated northward until reaching their modern location (Hewitt 1996). However, little is known about the postglacial history of subtropical or tropical tree species. It has been proposed that during the last glacial maximum, speciation was prompted in the tropical and subtropical lowlands

Correspondence: Juan P. Jaramillo-Correa, Fax: (+1) (418) 656-7493; E-mail: jpjc@rsvs.ulaval.ca; jpjc@gmx.net

due to isolation in refugia, contributing to the diversity currently observed in these zones (e.g. Pennington *et al*. 2004). On the other hand, the montane species would have expanded their ranges to lower altitudes during the cooler periods, and then retreated towards higher elevations until being confined to small island-like pockets of favourable habitat during the Holocene warming (e.g. Jackson *et al*. 1996). Such small isolated stands would be exposed to stochastic forces such as inbreeding or genetic drift, which could lead to a rapid loss of genetic variability and potential extinction. However, these remnant populations could also be seen as interglacial refugia from which species could re-expand during the next glacial cycle (Ledig *et al*. 2000a). Given their unusually high species diversity and endemism (Anonymous 1991), the forests of the Sierra Madre Occidental in northwestern México are ideal ecosystems to study the evolutionary impacts of the glacial cycles on subtropical montane species.

Spruce (*Picea* A. Dietr.) is a predominantly boreal or temperate conifer genus, which includes between 31 and 50 species depending on the classification system used (e.g. Wright 1955; Schmidt 1989; Farjon 2001). In the montane forests of Mexico, three rare and endemic spruce species occur in small relict populations between 2200 and 3500 m above sea level (Ledig *et al*. 2000b; Farjon 2001). While some of the boreal spruce species are among the most studied taxa at the ecological and genetic levels (Nienstaedt & Zasada 1990; Viereck & Johnston 1990), only a few ecological, genetic, and demographical data have been gathered on the Mexican taxa (e.g. Gordon 1968; Ledig *et al*. 1997, 2000a, b). The most common of the Mexican spruces is Chihuahua spruce (*Picea chihuahuana* Martínez), also known as prickly spruce. This taxon was first reported in 1942 from a site called Talayotes in the state of Chihuahua (Martínez 1953). Since then, 38 more stands have been reported in the Sierra Madre Occidental in both Chihuahua and its neighbouring Durango (Narváez *et al*. 1983; Ledig *et al*. 2000b). The species spans a north–south range of 687 km, but populations are found in three clusters, each separated by about 300 km. In total, there are about 43 000 individuals, including mature trees, saplings, and seedlings (Ledig *et al*. 2000b). These low demographic figures have represented a convincing case for the inclusion of *P. chihuahuana* in the lists of threatened species prepared by the International Union for the Conservation of Nature and Natural Resources (IUCN) and the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP) (Sánchez-Córdoba & Narváez-Flores 1990; Vera 1990).

As predicted for subtropical montane taxa (Jackson *et al*. 1996), the Mexican *Picea* had a broader distribution during the Pleistocene, which diminished considerably during the Holocene. Previous pollen-based population reconstructions have shown that 5 million years ago, during the mid-Pliocene, Mexican spruce populations (*Picea* spp.) could have occurred as far south as the Isthmus of Tehuantepec, 900 km from the southernmost modern spruce stands (Graham 1993). Consequently, the range reductions experienced by these taxa would have driven the remaining populations to bottlenecks, with concomitant inbreeding depression and genetic drift (Ledig *et al*. 1997, 2000a). However, further biogeographic studies are necessary to establish, at the intraspecific level, how the Holocene warming and the consequent vicariance modelled the population structure of each of the Mexican spruce species.

Biogeographic inferences are usually based on pollen and fossil records, but they generally have low taxonomical and morphological resolution at the intraspecific level and sometimes, at the intrageneric level. This has been the case for North American species of the genera *Picea* and *Pinus* (e.g. Davis 1983; Jackson *et al*. 1997). However, these biogeographic inferences have been improved during the recent years with the extensive use of molecular markers (e.g. Walter & Epperson 2001; Richardson *et al*. 2002; Jaramillo-Correa *et al*. 2004; Godbout *et al*. 2005). In the Pinaceae, the organelle genomes harbour contrasted inheritance, chloroplasts being paternally inherited and mitochondria maternally transmitted (e.g. Neale & Sederoff 1988). Such a discordant inheritance may allow the study of the effects of differential levels of gene flow between seed and pollen, and facilitate the inference of historical events affecting population genetic structure (e.g. Richardson *et al*. 2002; Burban & Petit 2003). In the present study, we used the variation observed in both mitochondrial (mtDNA) and chloroplast (cpDNA) genomes of *P. chihuahuana* to infer its recent postglacial history and its modern population structure. Such information should be valuable for the establishment of long-term conservation programmes. Among others, we addressed the following questions: (i) did the species develop a geographic structure for both mtDNA and cpDNA during its Holocene retreat; (ii) if so, are the three extant Chihuahua spruce population clusters genetically homogeneous, or do they represent genetically distinct interglacial refugia; (iii) does the population structure revealed for maternally inherited mtDNA differ from that observed for paternally inherited cpDNA; and (iv) is there any evidence of genetic isolation (i.e. lack of gene flow) within and among the three remnant clusters of *P. chihuahuana* stands?

Materials and Methods

Population sampling and DNA extraction

Cones were collected separately from 156 individuals of *Picea chihuahuana* distributed in 16 stands covering most of the latitudinal range of the species. Seeds were extracted after the cones opened and were stored at 1 °C until needed. Demographic and ecological parameters for these populations were reported elsewhere (Ledig *et al*. 2000b). One seed per

tree was dissected to extract the haploid megagametophyte, which is representative of the maternal genotype including both cytoplasmic genomes. Each megagametophyte was ground and total DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN). DNA concentration was measured with a GeneSpec spectrophotometer (MiraiBio).

Detection of mtDNA and cpDNA polymorphisms

An exploration panel of 16 individuals was assembled to conduct preliminary tests for detecting mtDNA and cpDNA polymorphisms in *P. chihuahuana*, at a rate of one individual per population sampled. For the screening of mtDNA polymorphism by DNA sequencing, a part of the DNA sample of each of these 16 individuals was pooled in equal amount as reported by Pelgas *et al*. (2004), and then amplified in a PTC-225 thermal cycler (MJ Research) using primers for 14 mitochondrial regions (Table 1). Some of these mtDNA regions had previously shown polymorphisms in other *Picea* species (Jeandroz *et al*. 2002; Jaramillo-Correa *et al*. 2003, 2004). Additionally, two more primer pairs were developed *de novo* for amplifying introns 1 and 2 of the gene *rps*3 based on published sequences of *Cycas revoluta* (Regina *et al*. 2005). The polymerase chain reactions (PCR) were performed as reported elsewhere (Jeandroz *et al*. 2002; Jaramillo-Correa *et al*. 2003, 2004; Regina *et al*. 2005), except for the annealing temperatures which were set as described in Table 1. PCR products were examined by gel electrophoresis (2% agarose in TAE), and those exhibiting one single DNA fragment were sequenced. Direct sequencing of both DNA strands for each gene was conducted with the dideoxynucleotide chain termination procedure using the appropriate amplification primers and a Sequenase GC-rich kit (Applied Biosystems). A polymorphism was inferred when a double-peak or a frameshift was observed in the chromatograms of the DNA pool (Pelgas *et al*. 2004). To verify that these

Table 1 Target regions, annealing temperatures, observed size of PCR products, conditions to detect polymorphism and number of variants detected at 16 mtDNA and 6 cpDNA loci in Chihuahua spruce (*Picea chihuahuana*)

	Annealing temperature (°C)	Size of PCR product (bp)	Polymorphism detection	Number of variants detected	Primer source
mtDNA locus					
*matR**	58	500	monomorphic	1	Jaramillo-Correa *et al*. (2003)
*mh*05	52	Multiple bands	—	—	Jeandroz *et al*. (2002)
*mh*09	62	250	monomorphic	1	Jeandroz *et al*. (2002)
*mh*10	56	1800	*Mse*I and *Mbo*II‡	2	Jeandroz *et al*. (2002)
*mh*33	55	220	monomorphic	1	Jeandroz *et al*. (2002)
*mh*35	52	No amplification	—	—	Jeandroz *et al*. (2002)
*mh*44	55	Multiple bands	—	—	Jeandroz *et al*. (2002)
nad1 (intron b/c)	58	~3000	monomorphic	1	Demesure *et al*. (1995)
nad3–rps12 (i.r.)†	58	350	monomorphic	1	Soranzo *et al*. (1999)
nad5 (intron 1)	62	890	monomorphic	1	Jaramillo-Correa *et al*. (2003)
nad5 (intron 4)	52	Multiple bands	—	—	Wu *et al*. (1998)
nad7 (intron 1)	55	880	monomorphic	1	Jaramillo-Correa *et al*. (2004)
*rps*3 (intron 1)§	58	~2000	monomorphic	1	Present study
*rps*3 (intron 2)§	58	~1500	monomorphic	1	Present study
SSU *rRNA* (V1 region)	64	410	monomorphic	1	Duff & Nickrent (1999)
SSU *rRNA* (V7 region)	58	Multiple bands	—	—	Duff & Nickrent (1999)
cpDNA locus					
*Pt*15169	55	115	monomorphic	1	Vendramin *et al*. (1996)
*Pt*26081	55	120–123	P.A.G.E.¶	4	Vendramin *et al*. (1996)
*Pt*30204	55	138–142	P.A.G.E.¶	3	Vendramin *et al*. (1996)
*Pt*36480	55	145	monomorphic	1	Vendramin *et al*. (1996)
*Pt*45002	55	No amplification	—	—	Vendramin *et al*. (1996)
*Pt*71936	55	125	monomorphic	1	Vendramin *et al*. (1996)

*Incorrectly referred to as *matR* intron 1 by Jaramillo-Correa *et al*. (2003).
†i.r., intergenic region.
‡Digestion with each restriction endonuclease and separation through 8% polyacrylamide gel.
§Primers developed herein (see Materials and Methods): *rps3*-1 Forward: 5′-CCGAATCGTAGTTCAGATCCA-3′; *rps3*-1 Reverse: 5′-GTGCAACGCCTCTGACATAA-3′; *rps3*-2 Forward: 5′-TTTGGCTTTCGTCTCGGTAG-3′; *rps3*-2 Reverse: 5′-CCCTCACTTCGTTTCGTTCT-3′. PCR conditions set as in Regina *et al*. (2005).
¶P.A.G.E., polyacryalmide gel electrophoresis in a Li-Cor sequencer.

double-peaks or frameshifts were not produced by artefacts during the sequencing process, the DNA pool sequences were duplicated and compared to the sequences obtained from a single individual. Thus, the presence of a double-peak or a frameshift in the DNA pool sequences and its absence in the single-individual sequences would be indicative of a single nucleotide polymorphism (SNP) or an indel, respectively (see Pelgas *et al*. 2004 for more details). The information from the DNA pool sequencing was then used to screen for restriction endonucleases that digest selectively at the SNP or indel positions. These cleaved amplified polymorphic sequence (CAPS) markers were a more affordable detection method and were used to genotype all the individuals sampled. The digestion products were separated through 8% polyacryalmide gels (e.g. Jaramillo-Correa *et al*. 2003, 2004).

For cpDNA, we used the DNA of the 16 individuals of the preliminary panel separately. Following conditions reported elsewhere (Vendramin *et al*. 1996), DNA was amplified with primers for six cpDNA regions containing microsatellites (Table 1), some of which previously showed polymorphism in *Picea abies* (L.) Karst. (Vendramin *et al*. 2000). PCR products were separated through denaturing polyacrylamide gels [6.5× Long Ranger acrylamide: bisacrylamide (BioWhittaker Molecular Applications); 7 M Urea and 1× TBE buffer] in a Li-Cor 4200 sequencer. The nature of the polymorphic microsatellites was confirmed by sequencing using the same conditions than for the mtDNA markers. The 156 individuals sampled were then genotyped for the polymorphic cpDNA markers.

Genetic data analysis

Single locus mtDNA genotypes were considered simultaneously to define multilocus mtDNA haplotypes (mitotypes). Similarly, the cpDNA markers were pooled in chloroplast haplotypes (chlorotypes). Observed numbers of mitotypes and chlorotypes and mitochondrial and chloroplast diversity estimates (H; equivalent to the expected heterozygosity, H_E, for diploid data; Weir 1996) were calculated for each population and group of populations (see Table 2 for population grouping). The program BOTTLENECK version 1.2 (Piry *et al*. 1999) was used to compare the cpDNA and mtDNA gene diversities (H_E) estimated from chlorotype and mitotype frequencies in each single population with that expected under a drift–mutation equilibrium (H_{eq}). A significantly higher H_E value in a given population would suggest that this stand had experienced a recent and significant size reduction (Piry *et al*. 1999). The single-population H_{eq} values were estimated from the observed number of haplotypes (k_O) by assuming both an infinite allele (IAM) and a stepwise (SMM) mutation model. mtDNA and cpDNA sequences were used to determine the evolutionary relationships among mitotypes and among chlorotypes with minimum spanning trees. These trees were generated with the software TCS (Clement *et al*. 2000). mtDNA sequences of Martínez spruce (*Picea martinezii* T.F. Patterson), another Mexican spruce from the distant Sierra Madre Oriental in the state of Nuevo León, were also included for comparative purposes (see Discussion). This taxa has been considered conspecific to *P. chihuahuana* by some authors (e.g. Farjon 2001), and despite that previous biochemical and genetic studies demonstrated its distinct taxonomical status (Taylor *et al*. 1994; Ledig *et al*. 2004), it was included to confirm its distinctiveness at the genetic level. We thus repeated the previous numerical analyses by considering the mtDNA sequences of four Martínez spruces sampled in the populations of La Tinaja and Cañón el Butano (Nuevo León) (see Ledig *et al*. 2000a, 2000b, for full description of populations).

Differentiation among populations of *P. chihuahuana* and among groups of populations according to geographical proximity was estimated with an analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) conducted with the program ARLEQUIN (Schneider *et al*. 2000). Alternative groupings of populations were also tested using a spatial analysis of molecular variance (SAMOVA; Dupanloup *et al*. 2002). This method aims to identify the grouping of stands that maximize the proportion of total genetic variance due to differences between groups of populations (F_{CT}). A simulated annealing process was performed with the software SAMOVA (http://cmpg.unibe.ch/software/samova), until obtaining the configuration of K groups exhibiting the largest F_{CT} value (see Dupanloup *et al*. 2002 for more details).

Population structure of *P. chihuahuana* was further analysed by comparing the G_{ST} value with the N_{ST} and R_{ST} values derived from mitotype and chlorotype data with the programs PERMUT and CPSSR, respectively (Pons & Petit 1996). Contrary to the last two statistics, G_{ST} estimates the differentiation among populations without considering the relatedness among mitotypes or chlorotypes. Thus, a significantly lower value than a G_{ST}, N_{ST} or R_{ST} value, would be indicative of a phylogeographical structure. These calculations were performed by considering the 16 populations independently and the groups of populations defined by geographical proximity (see Table 2). Isolation by distance was tested by regressing pairwise estimates of F_{ST} and the linear function $F_{ST}/(1 - F_{ST})$ against the geographic distance between localities (Rousset 1997). Mantel tests with 10 000 permutations were used to test the correlation between the matrices of genetic differentiation and geographic distances using ARLEQUIN. Finally, the possible postglacial history of *P. chihuahuana* was assessed by comparing our results with the available ecological, palynological, and demographic data (e.g. Gordon 1968; Graham 1993; Lozano-García *et al*. 1993; Metcalfe *et al*. 1997; Ledig *et al*. 2000b).

Table 2 Genetic diversity estimates and frequencies of the mitotypes and chlorotypes observed in 16 populations of Chihuahua spruce (*Picea chihuahuana*)

Population/Group of populations Latitude/Longitude/Elevation (m.a.s.l.)		*n*	*N*	mtDNA *nh*	mtDNA *H*	Mitotype frequency I	Mitotype frequency II	cpDNA *nh*	cpDNA *H*	Chlorotype frequency I	II	III	IV	V	VI	VII	VIII
Northern populations (Group 1)																	
1	Arroyo de Chachamori 28°39′N/108°16′W/2320	10	146	1	0	0	1	2	0.42	0.30	0	0.70	0	0	0	0	0
2	Mategoina II 28°06′N/107°48′W/2255	10	124	1	0	0	1	3	0.62	0.20	0.3	0.50	0	0	0	0	0
3	Arroyo Ancho 28°04′N/107°47′W/2250	10	127	1	0	0	1	4	0.58	0.20	0	0.60	0.10	0	0	0	0.10
4	El Ranchito 27°57′N/107°45′W/2220	10	217	1	0	0	1	2	0.18	0.10	0	0.90	0	0	0	0	0
5	El Realito 27°56′N/107°37′W/2300	8	587	1	0	0	1	4	0.64	0	0.50	0.10	0.30	0	0	0.10	0
6	Las Trojas 27°54′N/107°45′W/2395	10	874	1	0	0	1	3	0.54	0.60	0.10	0.30	0	0	0	0	0
7	El Pinabetal 27°46′N/107°41′W/2305	10	455	1	0	0	1	4	0.70	0.20	0	0.30	0.40	0.10	0	0	0
8	Napahuichi II 27°54′N/107°37′W/2340	10	209	1	0	0	1	3	0.56	0	0	0.60	0.20	0	0	0.20	0
9	Río Vinihueachi 27°45′N/107°42′W/2160	9	1785	1	0	0	1	4	0.70	0.40	0	0.10	0.20	0.30	0	0	
Mean		9.67	506.67	1	0	0	1	3.22	0.548	0.22	0.10	0.45	0.13	0.04	0	0.03	0.01
Central populations (Group 2)																	
10	Arroyo de la Quebrada — El Vergel 26°28′N/106°21′W/2730	10	877	1	0	1	0	3	0.54	0.10	0.60	0.30	0	0	0	0	0
11	Arroyo del Indio Ignacio 26°09′N/106°23′W/2600	10	2628	1	0	1	0	1	0	0	0	1	0	0	0	0	0
12	La Estancia — Agua Amarillo 26°01′N/106°27′W/2580	10	1195	1	0	1	0	3	0.66	0.30	0.40	0.30	0	0	0	0	0
13	Faldeo de Cebollitas 25°06′N/106°27′W/2450	10	172	1	0	1	0	1	0	0	1	0	0	0	0	0	0
Mean		10	1218	1	0	1	0	2.0	0.300	0.10	0.50	0.40	0	0	0	0	0
Southern populations (Group 3)																	
14	Arroyo de las Lagunas 23°31′N/104°37′W/2775	10	505	1	0	1	0	1	0	0	1	0	0	0	0	0	0
15	Arroyo del Infierno 23°30′N/105°26′W/2725	9	148	1	0	1	0	2	0.18	0	0	0.90	0	0	0.10	0	0
16	Arroyo de la Pista 23°20′N/104°45′W/2685	10	919	1	0	1	0	2	0.32	0	0.80	0.20	0	0	0	0	0
Mean		9.76	524	1	0	1	0	1.66	0.167	0	0.60	0.40	0	0	0.30	0	0
Grand mean		156	685.5	1	0	0.438	0.562	2.63	0.415	0.150	0.294	0.425	0.075	0.025	0.006	0.019	0.006
Standard deviation				0	0	—	—	1.09	0.262	—	—	—	—	—	—	—	—

m.a.s.l., metres above sea level; *n*, number of trees sampled; *N*, populations census of mature trees (see Ledig *et al*. 2000b for more details); *nh*, number of haplotypes (mitotypes or chlorotypes); *H*, mtDNA or cpDNA genetic diversity.

Results

Detection of polymorphisms

Amplification was successful with the two new primer pairs developed herein (*rps*3 introns 1 and 2) for *Picea chihuahuana* and for most of the species of a conifer panel used in a previous study on cross-species amplification of mtDNA regions (Jaramillo-Correa *et al*. 2003). However, no apparent intraspecific polymorphism was observed in these regions for any of the conifers tested.

In *P. chihuahuana*, only two single nucleotide polymorphisms were detected among the 16 mtDNA regions analysed by DNA pool sequencing (Table 1). Contrary to other spruce species previously studied (Jeandroz *et al*. 2002; Jaramillo-Correa *et al*. 2003), no indel polymorphism was observed among the 16 individuals of the exploratory panel. The two SNPs detected were located in the marker *mh*10, a nontranscribed region developed from a mtDNA genomic bank in *Picea abies* (Jeandroz *et al*. 2002). These two SNPs could be revealed by DNA restriction with the endonucleases *Mse*I and *Mbo*II, respectively (Table 1; Fig. 1A). The two markers were completely linked and resulted in two mitotypes. The first mitotype (I) was characterized by having a T at the positions 325 and 1030 of the *mh*10 gene, while the second mitotype (II) had a G at the same locations (Fig. 1A). The evolutionary relationship between these mitotypes was inferred with a minimum spanning tree (Fig. 2A). An intermediate mitotype was expected between the two observed types, but it was not detected among the 156 individuals sampled. Only one mitotype was observed among the *Picea martinezii* individuals surveyed. Sequence analysis in this taxon revealed seven substitutions in five different genes (*matR* intron 1, *mh*10, *nad1* intron b/c, *nad7* intron 1, and SSU *rRNA* V1 region) when compared to *P. chihuahuana* (Fig. 2A). Such polymorphisms from multiple mtDNA regions were also observed among different spruce species (Jaramillo-Correa *et al*. 2003), supporting the idea that *P. chihuahuana* and *P. martinezii* are distinct species.

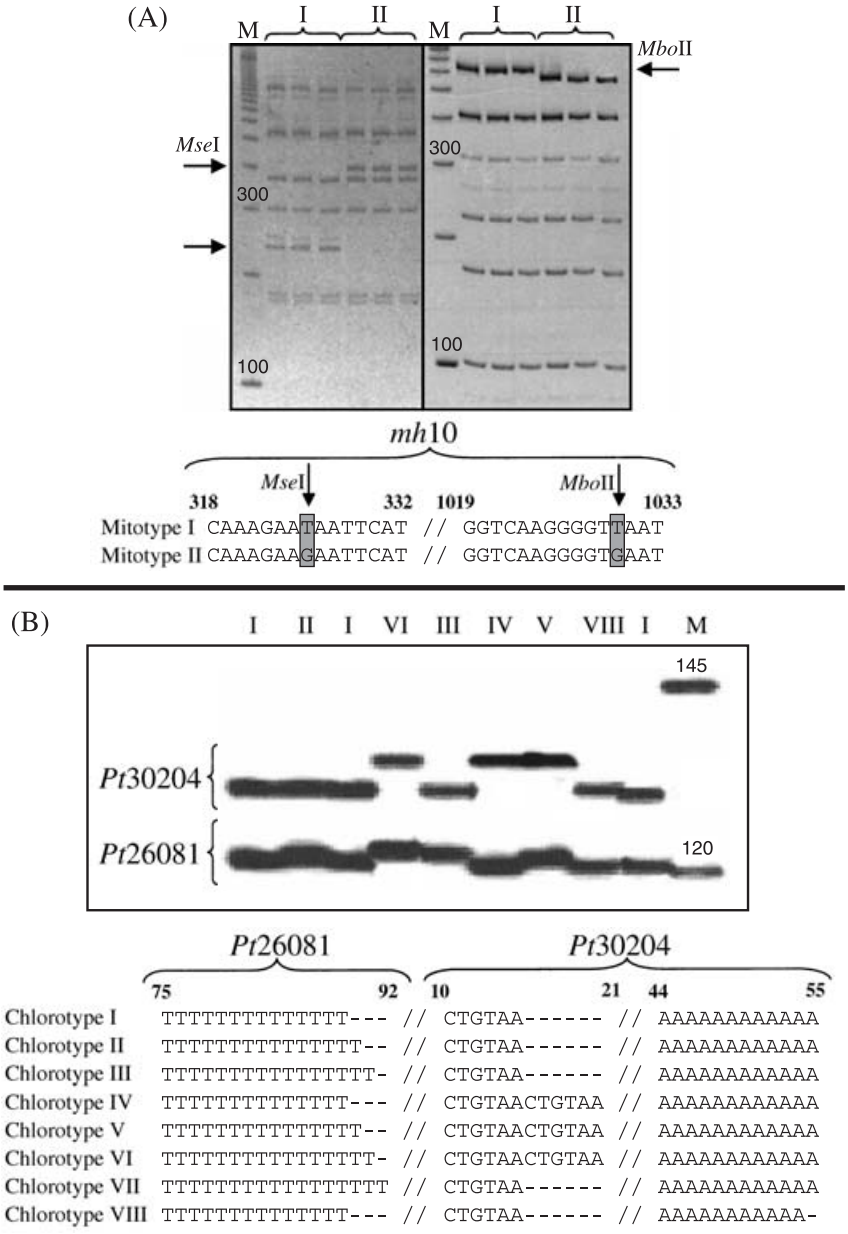

Fig. 1 Polymorphic mtDNA (A) and cpDNA (B) markers observed in Chihuahua spruce (*Picea chihuahuana*). Negative images of polyacrylamide gels and partial DNA sequences containing the polymorphic sites of each marker are shown. The molecular weight markers (M) used were a 100-bp ladder (Pharmacia) and a size standard IRDye 700 (Li-Cor), respectively. Indels are denoted by dashes and polymorphic restriction sites are shadowed.

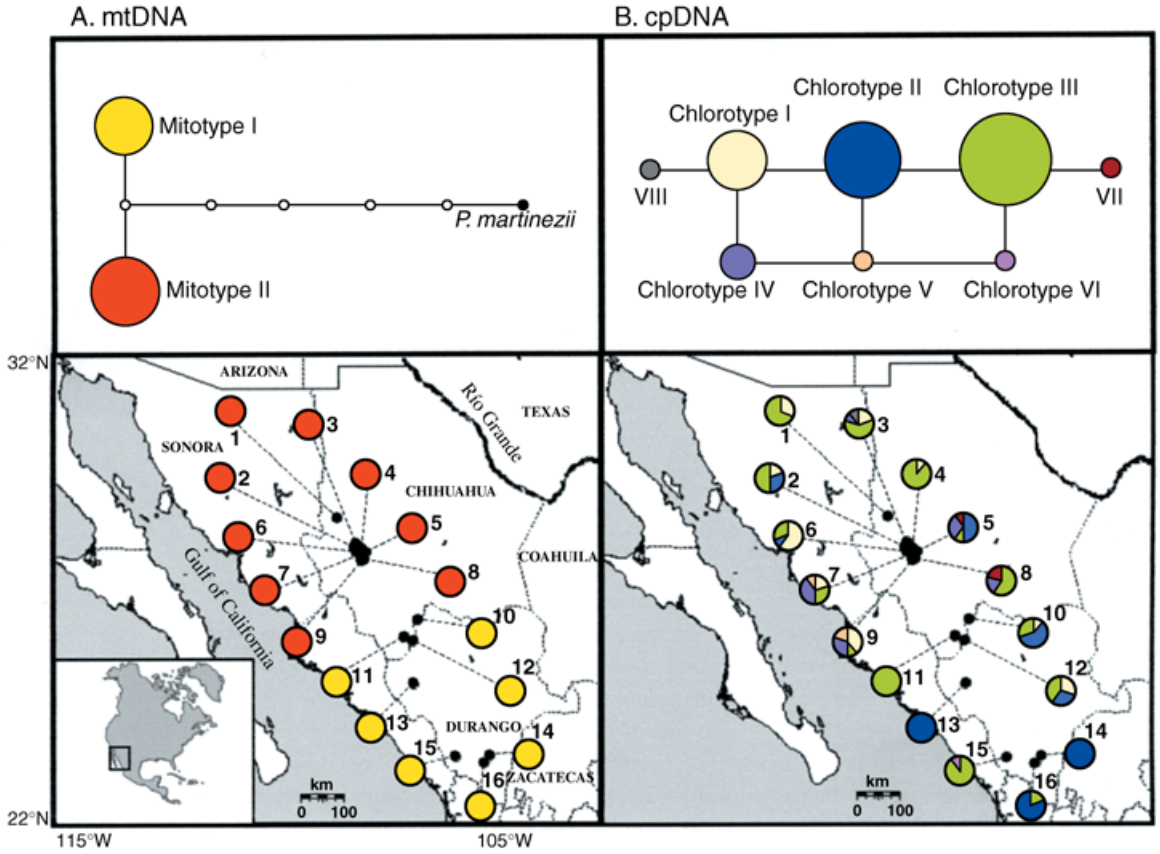

Fig. 2 Minimum spanning tree (top) and geographic distribution (bottom) of mitochondrial DNA (A) and chloroplast DNA haplotypes (B) in populations of Chihuahua spruce (*Picea chihuahuana*).

Among the six cpDNA regions surveyed, two (*Pt*26081 and *Pt*30204) revealed indel polymorphisms with the exploratory panel of 16 trees (Table 1; Fig. 1B). Sequence analysis further showed that the four variants observed for the marker *Pt*26081 were the product of a poly T microsatellite, which harboured between 14 and 17 repeats. Two of the three variants observed at the locus *Pt*30204 were also the product of a microsatellite, a poly A motif, repeated 11 or 12 times. The third variant of this locus was the product of a duplication of a 6-bp sequence located 23 bp upstream the microsatellite (Fig. 1B). No SNPs nor fragment length homoplasies were observed among the six chloroplast markers surveyed after extensive DNA sequencing of individuals from different populations (data not shown). Contrary to the mtDNA markers, the cpDNA markers did not appear to be linked and formed eight distinct chlorotypes (Fig. 1B), instead of the six that would have been expected if they were completely linked. The minimum spanning tree inferred from the chlorotype sequences did not allow to determine a single and clear evolutionary path as it contained some alternative links or loops between chlorotypes I and VI (Fig. 2B). These ambiguities could suggest recurrent mutation or even genomic recombination in the chloroplast genome (e.g. Marshall *et al*. 2001). The analysis of the 156 individuals did not reveal any further polymorphisms or additional chlorotypes. The sequences for some of the mtDNA and cpDNA regions surveyed for both *P. chihuahuana* and *P. martinezii* are available on GenBank (Accession nos DQ415963–DQ415984).

Distribution of mtDNA and cpDNA diversity

None of the 16 *P. chihuahuana* populations surveyed was polymorphic for the mtDNA markers (Table 2; Fig. 2A). The distribution of mitotypes revealed two clearly defined zones, one formed by the populations of northern Chihuahua (#1 to #9 in Table 2) which were all fixed for mitotype II, and a second group composed by the central (#10 to #13) and southern (#14 to #16) populations, which were all fixed for mitotype I (Fig. 2A). Although it cannot be estimated mathematically, the genetic differentiation between these two groups of populations fixed for different mitotypes was maximal ($G_{ST} = N_{ST} = 1$). No further statistical testing could be conducted with such a disconnected geographical pattern of variation.

For cpDNA markers, 3 of the 16 stands surveyed were fixed for a particular chlorotype. Populations #13 and #14

were fixed for chlorotype II and population #11 was fixed for chlorotype III (Table 2; Fig. 2B). The remaining populations bore between two and four cpDNA variants, and with some exceptions (populations #7 to #9), most of these stands bore one or two common chlorotypes and up to two rare cpDNA variants. There were four common and four rare chlorotypes among the eight types detected in *P. chihuahuana*. Chlorotype III was the most abundant form being present in all but two populations (#13 and #14). It was followed by chlorotype I which was present in the northern and central stands, by chlorotype II which was mostly found in the central and southern populations, and by chlorotype IV which was exclusive to some of the northern stands (#3, #5, #7, #8, and #9; Table 2; Fig. 2B). The four remaining chlorotypes (V, VI, VII and VIII) were all present in one or two populations and were rare, with mean frequencies below 5% (Table 2; Fig. 2B). Overall, the cpDNA diversity appeared to be low. In four stands (#3, #4, #5, #15), the estimated chlorotype diversity (H_E) was lower than that expected under the mutation–drift equilibrium (H_{eq}), suggesting a significant reduction of effective population size (i.e. a recent bottleneck) in these populations (Table 3).

Globally, the cpDNA diversity diminished from north to south ($r_{H,Lat} = 0.626$; $P < 0.01$). The northern populations had the highest cpDNA diversity ($nh = 3.22$; $H = 0.548$), followed by the central ($nh = 2.0$; $H = 0.300$) and the southern stands ($nh = 1.66$; $H = 0.167$. See Table 2). However, this general trend was not reflected in a phylogeographical structure, as the global G_{ST} value (0.362) was higher than the R_{ST} value (0.230). When the populations were gathered in three groups based on geographical proximity (see Table 2 and Fig. 2), the G_{ST} value (0.081) was lower than the R_{ST} value (0.102), but this difference was not significant ($P > 0.05$). A similar picture was obtained when the populations were gathered in the two groups depicted by the distribution of mitotypes ($G_{ST} = 0.159$; $R_{ST} = 0.184$; $P > 0.05$). The analysis of molecular variance (AMOVA) indicated that most of the cpDNA diversity was located within populations (70.47%), while the differentiation among the three previously defined geographic groups only accounted for 3% of the total variation (Table 4). When this analysis was repeated by considering the two contrasted groups of populations revealed by the distribution of mitotypes (Fig. 2A), differentiation among groups barely explained more than 5% of the cpDNA variation, while most of the diversity was still located within populations (Table 4).

The spatial analysis of molecular variance (SAMOVA) and the Mantel test further supported the absence of a geographic structure underlying cpDNA variation in *P. chihuahuana*. SAMOVA showed that only two genetically homogenous groups could be delineated from the distribution of chlorotypes (data not shown). These groups differed greatly from those delineated by the geographic distribution of the populations (Table 2) and from those depicted by mtDNA variation (Fig. 2A). One group was composed by the two populations with the greatest genetic diversity (#7 and #9; Table 2), while the other was formed by the remaining stands. It must be noted that populations #7 and #9 are located on branches

Table 3 Results from the tests of bottlenecks on chloroplast haplotype frequencies of 16 populations of Chihuahua spruce (*Picea chihuahuana*)

Population		n	k_O	H_E	H_{eq} IAM	H_{eq} SMM
1	Arroyo de Chachamori	10	2	0.420	0.357	0.383
2	Mategoina II	10	3	0.620	0.560	0.608
3	Arroyo Ancho	10	4	0.580	0.699*	0.736*
4	El Ranchito	10	2	0.180	0.349***	0.382***
5	El Realito	8	4	0.640	0.752***	0.772***
6	Las Trojas	10	3	0.540	0.562	0.614
7	El Pinabetal	10	4	0.700	0.699	0.737
8	Napahuichi II	10	3	0.560	0.566	0.603
9	Río Vinihueachi	9	4	0.700	0.727	0.751
10	Arroyo de la Quebrada–El Vergel	10	3	0.540	0.567	0.602
11	Arroyo del Indio Ignacio	10	1	0	—	—
12	La Estancia–Agua Amarillo	10	3	0.660	0.559	0.608
13	Faldeo de Cebollitas	10	1	0	—	—
14	Arroyo de las Lagunas	10	1	0	—	—
15	Arroyo del Infierno	9	2	0.180	0.368***	0.390***
16	Arroyo de la Pista	10	2	0.320	0.340	0.381

n, number of trees sampled; k_O, number of observed chlorotypes; H_E, estimated cpDNA diversity; H_{eq}, expected cpDNA diversity under a mutation–drift equilibrium by assuming an infinite allele (IAM) and a stepwise (SMM) mutation models; *$P < 0.05$; ***$P < 0.001$.

Table 4 Results from two analyses of molecular variance (AMOVA) of chloroplast haplotype frequencies considering two different groupings of 16 populations of Chihuahua spruce (*Picea chihuahuana*)

Source of variation	d.f.	SS	Variance components	Percentage of variation	*F*-statistics
Among three geographical groups†	2	9.98	0.033	3.17^{NS}	F_{CT} = 0.032^{NS}
Among populations within groups	13	44.89	0.272	26.36***	F_{SC} = 0.272***
Within populations	144	104.90	0.728	70.47***	F_{ST} = 0.295***
Total	159	159.77	1.034		
Among two mtDNA groups‡	1	7.64	0.054	5.17^{NS}	F_{CT} = 0.052^{NS}
Among populations within groups	14	47.23	0.265	25.26***	F_{SC} = 0.266***
Within populations	144	104.90	0.728	69.57***	F_{ST} = 0.304***
Total	159	159.77	1.047		

d.f., degrees of freedom; SS, sum of squares; ^{NS}, nonsignificant; ***$P < 0.001$.
†See Table 2 for group definitions.
‡See Fig. 2A for group definitions.

of the same river at only 2 km from each other, thus suggesting that they formed one continuous stand in the recent past (Ledig *et al*. 2000b). Based on this fact, we repeated the SAMOVA by considering these two populations as a single one, but the resulting groups and the associated F_{CT} value remained unchanged (data not shown). On the other hand, the Mantel test revealed a lack of correlation between both the pairwise estimates of F_{ST} and the linear function $F_{ST}/(1 - F_{ST})$ with the geographic distances among populations ($r_{F_{ST},\,Geo} = 0.168$; $P = 0.186$ and $r_{Lin,Geo} = -0.021$; $P = 0.572$ respectively), further suggesting a lack of similarity based on proximity (i.e. isolation and distance were not related). No significant correlation was observed when comparing the cpDNA diversity with the population census ($r_{H,\ln N} = 0.064$; $P = 0.863$) nor with any other ecological or demographic factors previously considered (Ledig *et al*. 2000b) in the *P. chihuahuana* populations surveyed herein (data not shown).

Discussion

Amounts and distribution of mtDNA and cpDNA diversity

In plants, the mutation rates of the mitochondrial genome are generally lower than those observed in their chloroplast and especially, in their nuclear counterparts (Wolfe *et al*. 1987; Laroche *et al*. 1997). These low mutation rates usually translate into a low mtDNA variation at the inter- and intraspecific levels (e.g. Latta & Mitton 1997; Burban & Petit 2003; Gamache *et al*. 2003; Jaramillo-Correa *et al*. 2003; Gros-Louis *et al*. 2005). This trend was also noted in *Picea chihuahuana*. In this species, only two substitution polymorphisms were detected in one of the 16 mtDNA regions screened by DNA pool sequencing. When compared with other spruces such as *Picea mariana* or *Picea abies*, the levels of mtDNA diversity of *P. chihuahuana* appeared to be rather low. Most of the 16 mtDNA regions analysed here have been used previously to survey the mtDNA variation in these two species, revealing between 5 and 11 mitotypes (Bastien *et al*. 2003; Jaramillo-Correa *et al*. 2003, 2004). However, the amount of mtDNA variation detected in *P. chihuahuana* was similar to that reported in other congeneric species such as *Piceaglauca*, *Picea pungens*, *Picea sitchensis* and *Picea jezoensis* (*nh* = 2; Jaramillo-Correa *et al*. 2003) and in other conifers from the genus *Pinus* and *Abies* (*nh* = 2 to 3; *H* = 0 to 0.10; Latta & Mitton 1997; Liepelt *et al*. 2002; Burban & Petit 2003). To date, most of the mtDNA polymorphisms observed in conifers were due to indels, and in some cases to variation in repetitive patterns (i.e. minisatellites; Bastien *et al*. 2003; Godbout *et al*. 2005). In *P. chihuahuana*, no indels nor repetitive patterns were detected despite of the intensive sequence analysis employed. Complementary DNA analysis such as restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphim (SSCP) of the 16 mtDNA regions surveyed failed to reveal additional variation (data not shown), confirming the picture obtained by DNA pool sequencing.

The eight chlorotypes detected by the analysis of six microsatellite loci and the cpDNA diversity estimated in *P. chihuahuana* (*H* = 0.42) represent much lower values than those previously reported for a similar set of cpDNA microsatellites in more northern conifers (*nh* = 15–41; *H* = 0.80 to 0.85; Vendramin *et al*. 1999, 2000; Clark *et al*. 2000; Burban & Petit 2003), or than those observed in the southern relict *Pinus canarensis* (*nh* = 27; *H* = 0.73; Gómez *et al*. 2003), a species endemic to the Canary Islands. However, it was comparable to that detected in *Pinus resinosa* (*nh* = 6; *H* = 0.14; Walter & Epperson 2001), a temperate North American conifer which has apparently suffered an extreme bottleneck during the last glaciation (Fowler & Morris 1977). The small amounts of mtDNA and cpDNA diversity (Table 2), and the significant population size

reduction detected in some stands (Table 3), together with previous estimates of nuclear gene diversity based on allozymes (Ledig *et al*. 1997), suggest that *P. chihuahuana* has been through strong bottlenecks and suffered from genetic drift during the recent past, similar to the situation in *P. resinosa*.

Picea chihuahuana populations seemed to be less differentiated for cpDNA (G_{ST} = 0.362) than for mtDNA markers (G_{ST} = 1). The distribution of the maternally inherited mitotypes indicated that the populations of this species could be divided into two distinct groups represented by the northern (populations #1 to #9), and the south and central (#10 to #16) stands, respectively (Fig. 2A). The distribution and the spatial analyses of the paternally inherited chlorotypes did not reveal such a clear population structure (Fig. 2B; Table 4), showing that most of the cpDNA variation was located within populations. Such a lack of geographic structure was also observed for nuclear allozyme loci (Ledig *et al*. 1997).

In conifers, variation in the mitochondrial genome represents the gene flow mediated by seed, while variation in the chloroplast genome describes gene flow attributable to both pollen and seed (Burban & Petit 2003). Given the absence of intrapopulation variation detected for mtDNA, it would be safe to assume that gene flow through seed between the two contrasted groups of populations based on mtDNA would be negligible. On the other hand, the gene flow for haploid cpDNA markers could be estimated by applying the formula $Nm = [(1 - F_{ST})/2F_{ST}]$ (Takahata & Palumbi 1985). If we assume that G_{ST} and F_{ST} are equivalent estimates of population differentiation, the mean G_{ST} value estimated from chlorotype frequencies for the 16 *P. chihuahuana* populations would translate to a mean gene flow (*Nm*) of 0.87 migrants per generation. Similarly, cpDNA gene flow between the two contrasting groups of populations defined by mtDNA variation was estimated as 2.63 migrants per generation (Fig. 2A). These estimates of gene flow are very low when compared to those observed in other conifers (e.g. Latta & Mitton 1997; Burban & Petit 2003; Gamache *et al*. 2003), but they confirm a general trend for this group of plants, namely that gene flow mediated by pollen is higher than gene flow prompted by seed dispersal.

Low estimates of gene flow were also obtained in a previous study with nuclear allozymes (Ledig *et al*. 1997). In their study, the authors concluded that the gene flow among populations was so low that populations might have been effectively isolated for at least 40 generations (Ledig *et al*. 1997). These inferences are supported by the lack of isolation by distance revealed by the Mantel test based on cpDNA variation observed herein. The absence of correlation between the matrix of pairwise genetic differentiation values and that of geographic distances indicates that gene flow among populations was not related to the geographic distance among them. A striking example would be the comparison of populations from El Ranchito (#4 in Table 2) and Las Trojas (#6), which are separated by less than 6 km. These two stands had a very different chlorotype composition (Table 2; Fig. 2B), which was associated with a pairwise F_{ST} value of 0.436 (data not shown). This value represents 0.65 migrants per generation, which is an extremely low migration rate for a wind-pollinated conifer. Such low gene flow could be explained by the isolated nature of the *P. chihuahuana* stands: they are almost always located in narrow riparian strips on the north slopes of steep-walled arroyos (Ledig *et al*. 2000b). This is in sharp contrast with some largely distributed boreal spruces where levels of nuclear gene flow are more than one order of magnitude higher (e.g. Gamache *et al*. 2003).

One might argue that selection instead of historical and demographic factors could be responsible for the high population differentiation observed in *P. chihuahuana* at various levels, between regions for mtDNA and among stands for cpDNA. However, it is doubtful that selective forces would affect strongly markers located in noncoding regions of cytoplasmic genomes such as those used in the present study. Moreover, no correlation was observed when comparing the cpDNA variation with the ecological and demographic factors previously determined (Ledig *et al*. 2000b) in the *P. chihuahuana* populations studied (data not shown). A similar analysis with nuclear allozyme loci (Ledig *et al*. 1997) revealed that the nuclear genetic diversity of *P. chihuahuana* was more related to population size than to any other geographical or ecological feature. Such a trend is a clear indication of the involvement of genetic drift as the main determinant of population diversity in Chihuahua spruce instead of selection.

Fossil and molecular evidence for a Holocene retreat of P. chihuahuana

Although the proportion of *Picea* spp. pollen in the Mexican sediment cores is usually low, its distribution suggests that these taxa were more abundant and widespread in the past (Graham 1993; Lozano-García *et al*. 1993; Metcalfe *et al*. 1997; Caballero *et al*. 1999), with presumed occurrence as far south as the Isthmus of Tehuantepec, more than 900 km from the southernmost modern spruce populations (Graham 1993). According to different sediment cores from the basin of México (central México), *Picea* spp. also occurred in this region about 40 000 years before present (BP) (Caballero *et al*. 1999). These spruce populations would have expanded locally until the end of the Pleistocene, 12 500–9000 years BP, and then declined until they disappeared entirely from the region about 7000–8000 years BP when climate was warming (Lozano-García *et al*. 1993). Similarly, sediment cores of the Alta Babícora basin in the state of Chihuahua indicated that *Picea* spp. was also abundant in this area

before the last glacial maximum, and then became scarce during the last 11 000 years (Metcalfe *et al*. 1997). Most of the estimated dates for the retreat of *Picea* spp. in México coincide roughly with the beginning of the Holocene, which conduced to a more arid period in this country (Heine 1973).

The topographic and botanical features of México suggest that migration of montane plant species from central México to the Sierra Madre Occidental is apparently more likely than migration from the basin of México to the Sierra Madre Oriental (McDonald 1993). The particular flora and entomofauna of the Sierra Madre Oriental further suggests that this mountain range was not connected, during the Pleistocene, with the Transverse Volcanic Belt in which the basin of México is located (Halffter 1987; McDonald 1993). Of the three Mexican spruce species, *P. chihuahuana*, *Picea martinezii*, and *Piceamexicana*, only the first one is now found in the Sierra Madre Occidental, while the last two occur in the Sierra Madre Oriental. Consequently, it could be inferred safely that the pollen and macrofossils found in the Basin of México are most likely *P. chihuahuana*, and that this species has retreated about 500 km to the north during the last 8000 years. Another line of evidence suggesting the southern presence of *P. chihuahuana* is its tolerance to higher temperatures when compared to other spruces, including *P. mexicana* and *P. martinezii*. In growth chamber experiments, *P. chihuahuana* was able to survive under temperatures that would normally kill the other species (Ledig *et al*., unpublished).

The distribution of mtDNA haplotypes suggests that the modern relict populations of *P. chihuahuana* originate from two distinct populations in the near past (Fig. 2A). Once geographically divided, the ancestral populations would have become isolated in terms of gene flow by seed, and then become fixed for different mitotypes following genetic drift. These ancestral stands would have been further fragmented in a relatively short period of time at the geological scale, increasing the action of genetic drift and inbreeding within each remaining population.

The modern distribution of chlorotypes further suggests that the two ancestral populations might have continued to exchange pollen after being geographically separated. For instance, only a few populations became fixed for a particular chlorotype, while some of the larger stands (i.e. #9) kept a moderate cpDNA diversity (Table 2). Gene flow mediated by pollen among relict populations or refugia has been also inferred in other northern conifers (e.g. Liepelt *et al*. 2002; Richardson *et al*. 2002; Burban & Petit 2003). However, the *Nm* estimates derived from cpDNA (present study) and allozyme variation (Ledig *et al*. 1997), together with the pairwise F_{ST} values (see above) indicate that, as the two ancestral populations became further fragmented, the gene flow mediated by pollen diminished until effectively isolating (*Nm* below 1) some of the *P. chihuahuana* stands (Ledig *et al*. 1997).

Evolutionary relationship between P. chihuahuana *and* P. martinezii

Some authors have suggested that the spruce populations from Nuevo León, nowadays called *P. martinezii*, do not represent a distinct species but are conspecific to *P. chihuahuana* (e.g. Farjon 2001). However, the molecular data from the present and previous studies suggest otherwise. The comparative analysis of mtDNA sequences indicates that the spruce populations from Nuevo León (*P. martinezii*) are different enough from *P. chihuahuana* to be considered as a different taxon (Fig. 2A). Indeed, the number of fixed mtDNA polymorphisms found between the two taxa is in the range of that observed between *P. abies*, *P. glauca*, and *P. mariana* (Jaramillo-Correa *et al*. 2003), three reproductively isolated and phylogenetically divergent species in the genus (Wright 1955; Sigurgeirsson & Szmidt 1993). Such sizeable divergence between *P. chihuahuana* and *P. martinezii* was also observed with a large set of nuclear and cpDNA markers (Ledig *et al*. 2004), and further confirmed by sequence analysis of three cpDNA regions (Bouillé & Bousquet, unpublished). Previous morphological and biochemical analyses further suggested that these two taxa are different enough to warrant the status of distinct species (Taylor *et al*. 1994). Only six populations of *P. martinezii* have been reported (Ledig *et al*. 2000b), and the low levels of genetic diversity observed at nuclear allozyme loci (Ledig *et al*. 2000a), together with the complete lack of variation noted at mtDNA and cpDNA loci surveyed in the present study (data not shown), confirm the critical status of this species at the genetic level.

Considerations for the conservation of P. chihuahuana

In recent years, conservation strategies for threatened tree species have relied increasingly on genetic and phylogeographic data (Newton *et al*. 2003). The allelic richness and the variance in allele frequencies observed among natural populations have been shown to be particularly useful to estimate the contribution of each population to the total genetic diversity, and to determine the location of potential refugial areas for conservation purposes (Petit *et al*. 1998; Newton *et al*. 2003). In the present study, we determined that the northern populations of *P. chihuahuana* bore higher cpDNA diversity than the central and southern stands (Table 2). The distribution of diversity at nuclear allozyme loci appeared to follow a similar pattern (Ledig *et al*. 1997), while a previous ecological study (Ledig *et al*. 2000b) further revealed that the southern populations contained larger and probably older trees which were less affected by spruce broom rust (incorrectly referred to as mistletoe in Ledig *et al*. 2000b) than those from the northern and central stands. However, natural regeneration seems to be more abundant in these northern and central populations than in the southern

ones. These different trends suggest that the populations in the north are younger and could survive longer without human assistance (Ledig *et al*. 2000b), and that the southern populations may require greater conservation efforts than the northern ones.

It has also been suggested that large populations and populations with high levels of diversity should be prioritized for *in situ* conservation because of their presumed increased capacity for adaptation and for favouring the ecosystem recovery after drastic environmental changes (Reusch *et al*. 2005). Consequently, given the evidence that genetic drift, not selection, has largely contributed to the differentiation of most *P. chihuahuana* populations, it is possible that several northern stands, especially those exhibiting high levels of genetic diversity are also worthy of conservation. Population size may not always be a good predictor for designing conservation areas in *P. chihuahuana*, and some smaller stands may also require protection because of the high levels of differentiation among populations. For example, the conservation of northern or southern populations alone would certainly result in the loss of a mitotype and a significant part of the observed chlorotype variation. *Ex situ* conservation strategies such as the propagation of plants derived from tissue culture and *in vitro* regeneration (e.g. Fay 1994; Mata Rosas *et al*. 2001) should be reconsidered for *P. chihuahuana*, as they are more expensive and do not capture all of the genetic diversity already present in natural populations.

Any attempt to restore and enhance gene flow among populations or to design seed zones for reforestation should take into account the population structure detected in this and previous genetic studies (Ledig *et al*. 1997). In particular, the precautionary principle suggests that the novel portrait depicted by mtDNA markers should be taken into account even if we have no evidence of variation in quantitative characters between the two mitotype groups. For instance, seed transfer from the northern to the central or southern stands and vice versa should be avoided. Further studies including additional sampling and the assessment of quantitative traits appear necessary in order to better determine the adaptive value of particular phenotypic traits and to compare the genetic structure of adaptive characters to that derived from neutral genetic markers.

Acknowledgements

This study was a task of the Forest Genetic Resources Working Group/North American Forest Commission/Food and Agricultural Organization of the United Nations. We are grateful to T. Eguiluz-Piedra, C. Flores-López, M. Mápula-Larreta, and the late B. Bermejo-Velázquez for help in planning and sampling *P. chihuahuana* populations. We also thank D.R. Johnson for assistance in the field, E. Pouliot, M. Deslauriers, J.L. Jany, and S. Senneville for valuable help in the laboratory, and M. Bouillé for sharing her unpublished DNA sequence data and for fruitful discussions on phylogenetic issues. Helpful comments from R.J. Petit, I. Gamache and two anonymous reviewers on earlier drafts of the manuscript are also acknowledged. This research was supported by funding from the Canadian Forest Service to J. Be and a grant from the National Science and Engineering Research Council of Canada and a Canada Research Chair to J. Bo. Seed collections were funded with the help of USDA Office of International Cooperation and Development project no. 190-6 and US National Research Initiatives Competitive Grant program award no. 95-37101-1916 to F.T.L.

References

Anonymous. (1991) NS/S nominates Sierra Madre as a global center of plant diversity. *The Seedhead News*. No. **34**, 4. Native Seeds/SEARCH, Tucson, Arizona.

Bastien D, Favre JM, Collingnon AM, Sperisen C, Jeandroz S (2003) Characterization of a mosaic minisatellite locus in the mitochondrial DNA of Norway spruce [*Picea abies* (L.) Karst]. *Theoretical and Applied Genetics*, **107**, 574–580.

Burban C, Petit RJ (2003) Phylogeography of maritime pine inferred with organelle markers having contrasted inheritance. *Molecular Ecology*, **12**, 1487–1495.

Caballero M, Lozano S, Ortega B, Urrutia J, Macías JL (1999) Environmental characteristics of Lake Tecocomulco, northern basin of México, for the last 50 000 years. *Journal of Paleolimnology*, **22**, 399–411.

Clark CM, Wentworth TR, O'Malley DM (2000) Genetic discontinuity revealed by chloroplast microsatellites in eastern North American *Abies* (Pinaceae). *American Journal of Botany*, **87**, 774–782.

Clement MD, Posada DK, Crandall K (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.

Davis M (1983) Quaternary history of deciduous forests of eastern North America and Europe. *Annals of the Missouri Botanical Garden*, **70**, 550–563.

Demesure B, Sodzi N, Petit RJ (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology*, **4**, 129–131.

Duff RJ, Nickrent DL (1999) Phylogenetic relationships of land plants using mitochondrial small-subunit rDNA sequences. *American Journal of Botany*, **86**, 372–386.

Dupanloup I, Schneider S, Excofier L (2002) A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology*, **11**, 2571–2581.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Farjon A (2001) *World Checklist and Bibliography of Conifers*, 2nd edn. Royal Botanical Gardens, Kew, UK.

Fay MF (1994) In what situations is *in vitro* culture appropriate to conservation? *Biodiversity and Conservation*, **3**, 176–187.

Fowler DP, Morris RW (1977) Genetic diversity in red pine: evidence of low genetic heterozygosity. *Canadian Journal of Forest Research*, **7**, 343–347.

Gamache I, Jaramillo-Correa JP, Payette S, Bousquet J (2003) Diverging patterns of mitochondrial and nuclear DNA diversity in subarctic black spruce: imprint of a founder effect associated with postglacial colonization. *Molecular Ecology*, **12**, 891–901.

Godbout J, Jaramillo-Correa JP, Beaulieu J, Bousquet J (2005) A mitochondrial DNA minisatellite reveals the postglacial history of jack pine (*Pinus banksiana*), a broad-range North American conifer. *Molecular Ecology*, **14**, 3497–3512.

Gómez A, González-Martínez SC, Collada C, Clement J, Gil L (2003) Complex population genetic structure in the endemic Canary Island pine revealed using chloroplast microsatellite markers. *Theoretical and Applied Genetics*, **107**, 1123–1131.

Gordon AG (1968) Ecology of *Picea chihuahuana* Martínez. *Ecology*, **49**, 880–896.

Graham A (1993) Historical factors and biodiversity in México. In: *Biological Diversity of México: Origins and Distribution* (eds Ramamoorthy TP, Bye R, Lot A, Fa J), pp. 109–127. Oxford University Press, New York.

Gros-Louis MC, Bousquet J, Pâques LE, Isabel N (2005) Species diagnostic markers in *Larix* spp. based on RAPDs and nuclear, cpDNA, and mtDNA gene sequences, and their phylogenetic implications. *Tree Genetics and Genomes*, **1**, 50–63.

Halffter G (1987) Biogeography of the montane entomofauna of Mexico and Central America. *Annual Review of Entomology*, **32**, 95–114.

Heine K (1973) Variaciones mas importantes del clima durante los últimos 40,000 años en México. Communicaciones. Proyecto Puebla-Tlaxcala (Fundación Alemana de Investigación Científica), 7, 51–58.

Hewitt GM (1996) Some genetic consequences if ice ages, and their role in divergence and speciation. *Biological Journal of the Linnaean Society*, **58**, 247–276.

Jackson JBC, Budd AF, Coates AG, eds. (1996) *Evolution and Environment in Tropical America*. University of Chicago Press, Chicago, Illinois.

Jackson ST, Overpeck JT, Webb T III, Keattch SE, Anderson KH (1997) Mapped plant-macrofossil and pollen records of late Quaternary vegetation change in eastern North America. *Quaternary Science Reviews*, **16**, 1–70.

Jaramillo-Correa JP, Bousquet J, Beaulieu J *et al.* (2003) Cross-species amplification of mitochondrial DNA sequence-tagged-site markers in conifers: the nature of polymorphism and variation within and among species in *Picea*. *Theoretical and Applied Genetics*, **106**, 1353–1367.

Jaramillo-Correa JP, Beaulieu J, Bousquet J (2004) Variation in mitochondrial DNA reveals multiple distant glacial refugia in black spruce (*Picea mariana*), a transcontinental North American conifer. *Molecular Ecology*, **13**, 2735–2747.

Jeandroz S, Bastien D, Chandelier A, Du Jardin P, Favre JM (2002) A set of primers for amplification of mitochondrial DNA in *Picea abies* and other conifer species. *Molecular Ecology Notes*, **2**, 389–392.

Laroche J, Li P, Maggia L, Bousquet J (1997) Molecular evolution of angiosperm mitochondrial introns and exons. *Proceedings of the National Academy of Sciences, USA*, **94**, 5722–5727.

Latta RG, Mitton JF (1997) A comparison of population differentiation across four classes of gene markers in limber pine (*Pinus flexilis* James). *Genetics*, **146**, 1153–1163.

Ledig FT, Jacob-Cervantes V, Hodgskiss PD, Eguiluz-Piedra T (1997) Recent evolution and divergence among populations of a rare Mexican endemic, Chihuahua spruce, following Holocene climatic warming. *Evolution*, **51**, 1815–1827.

Ledig FT, Bermejo-Velázquez B, Hodgskiss PD *et al.* (2000a) 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. *Canadian Journal of Forest Research*, **30**, 1156–1164.

Ledig FT, Mápula-Larreta M, Bermejo-Velázquez B *et al.* (2000b) Locations of endangered spruce populations in México and the demography of *Picea chihuahuana*. *Madroño*, **47**, 71–88.

Ledig FT, Hodgskiss PD, Krutovskii KV, Neale DB, Eguiluz-Piedra T (2004) Relationships among the spruces (*Picea*, Pinaceae) of southwestern North America. *Systematic Botany*, **39**, 275–295.

Liepelt S, Bialozyt R, Ziegenhagen B (2002) Wind-dispersed pollen mediates postglacial gene flow among refugia. *Proceedings of the National Academy of Sciences, USA*, **99**, 14590–14594.

Lozano-García MS, Ortega-Guerrero B, Caballero-Miranda M, Urrutia-Fucugauchi J (1993) Late Pleistocene and Holocene paleoenvironments of Chalco Lake, central México. *Quaternary Research*, **40**, 332–342.

Marshall HD, Newton C, Ritland K (2001) Sequence-repeat polymorphisms exhibit the signature of recombination in lodgepole pine chloroplast DNA. *Molecular Biology and Evolution*, **18**, 2136–2138.

Martínez M (1953) *Las Pináceas Mexicanas*. Subsecretería de Recursos Forestales y de Caza, Secretaría de Agricultura y Ganadería, México.

Mata Rosas M, Chávez-Avila VM, Boettler RB (2001) *In vitro* regeneration of plantlets from immature zygotic embryos of *Picea chihuahuana* Martínez, an endemic Mexican endangered species. *In Vitro Cellular and Developmental Biology*, **37**, 73–78.

McDonald JA (1993) Phytogeography and history of the alpine-subalpine flora of northeastern México. In: *Biological Diversity of Mexico: Origins and Distribution* (eds Ramamoorthy TP, Bye R, Lot A, Fa J), pp. 681–703. Oxford University Press, New York.

Metcalfe SE, Bimpson A, Courtice AJ, O'Hara SL, Taylor DM (1997) Climate change at the monsoon/Westerly boundary in Northern Mexico. *Journal of Paleolimnology*, **17**, 155–171.

Narváez F, Sánchez RJ, Olivas AM (1983) *Distribución y población de Picea chihuahuana*. Secretaría de Agricultura y Recursos Hidráulicos (SARH), Insituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Centro de Investigaciones Forestales Norte, Nota Técnica no. 6. Matamoros, México.

Neale DB, Sederoff RR (1988) Inheritance and evolution of conifer organelle genomes. In: *Genetic Manipulation of Woody Plants* (eds Hanover J, Kethley D), pp. 251–264. Plenum Press, New York.

Newton AC, Allnutt TR, Gillies ACM, Lowe AJ, Ennos RA (2003) Molecular phylogeography, intraspecific variation and the conservation of tree species. *Trends in Ecology & Evolution*, **14**, 140–145.

Nienstaedt H, Zasada JC (1990) *Picea glauca* (Moench) Voss, white spruce. In Silvics of North America. vol. 1. Conifers. *Agricultural Handbook*, **654**, 227–237.

Pelgas B, Isabel N, Bousquet J (2004) Efficient screening for expressed sequence tag polymorphisms (ESTPs) by DNA pool sequencing and denaturing gradient gel electrophoresis (DGGE) in spruces. *Molecular Breeding*, **13**, 263–279.

Pennington TR, Lavin M, Prado DE *et al.* (2004) Historical climate change and speciation: neotropical seasonally dry forest plants show patterns of both Tertiary and Quaternary diversification. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **359**, 515–537.

Petit RJ, El Mousadik A, Pons O (1998) Identifying populations for conservation on the basis of genetic markers. *Conservation Biology*, **12**, 844–855.

Piry S, Luikart G, Cornuet J-M (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity*, **90**, 502–503.

Pons O, Petit RJ (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. *Genetics*, **144**, 1237–1245.

Regina TM, Picardi E, López L, Pesole G, Quagliariello C (2005) A novel additional group II intron distinguishes the mitochondrial *rps3* gene in gymnosperms. *Journal of Molecular Evolution*, **60**, 196–206.

Reusch TB, Ehlers A, Hämmerli A, Worm B (2005) Ecosystem recovery after climatic extremes enhanced by genotypic diversity. *Proceedings of the National Academy of Sciences, USA*, **102**, 2826–2831.

Richardson BA, Brunsfeld SJ, Klopfenstein NB (2002) DNA from bird-dispersed seed and wind-disseminated pollen provides insights into postglacial colonization and population genetics structure of whitebark pine (*Pinus albicaulis*). *Molecular Ecology*, **11**, 215–227.

Rousset F (1997) Genetic differentiation and estimation of gene flow from *F*-statistics under isolation-by-distance. *Genetics*, **145**, 1219–1228.

Sánchez-Córdoba J, Narváez-Flores R (1990) Plan integral para la protección y fomento de *Picea chihuahuana* Martínez. *Ecosistemas Forestales*. Instituto Nacional de Investigaciones Forestales y Agropecuarias. Secretaría de Agricultura y Recursos Hidráulicos, 1, 37–55.

Schmidt PA (1989) Beitrag zur Systematik und Evolution der Gattung *Picea* A. Dietr. *Flora, Jena*, **182**, 435–461.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN, version 2.000: A software for population genetics data analysis*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Switzerland.

Sigurgeirsson A, Szmidt AE (1993) Phylogenetic and biogeographic implications of chloroplast DNA variation in *Picea*. *Nordic Journal of Botany*, **13**, 233–246.

Soranzo N, Provan J, Powell W (1999) An example of microsatellite length variation in the mitochondrial genome of conifers. *Genome*, **42**, 158–161.

Takahata S, Palumbi SR (1985) Extranuclear differentiation and gene flow in the infinite island model. *Genetics*, **109**, 441–457.

Taylor RJ, Patterson TF, Harrod RJ (1994) Systematics of spruce — revisited. *Systematic Botany*, **19**, 47–59.

Vendramin GG, Lelli L, Rossi P, Morgante M (1996) A set of primers for the amplification of 20 chloroplast microsatellites in Pinaceae. *Molecular Ecology*, **5**, 111–114.

Vendramin GG, Degen B, Petit RJ *et al.* (1999) High levels of variation at *Abies alba* chloroplast microsatellite loci in Europe. *Molecular Ecology*, **8**, 1117–1126.

Vendramin GG, Anzidel M, Madahiele A, Sperisen C, Bucci G (2000) Chloroplast microsatellite analysis reveals the presence of population subdivision in Norway spruce (*Picea abies* K.). *Genome*, **43**, 68–78.

Vera G (1990) *Algunas especies arbóreas raras amenazadas en México*. Secretaría de Agricultura y Recursos Hidráulicos (SARH), Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), México.

Viereck LA, Johnston JW (1990) Black spruce. In Silvics of North America. vol. 1. Conifers. *Agricultural Handbook*, **654**, 227–237.

Walter R, Epperson BK (2001) Geographic pattern of genetic variation in *Pinus resinosa*: area of greatest diversity is not the origin of postglacial populations. *Molecular Ecology*, **10**, 103–111.

Weir BS (1996). *Genetic Data Analysis II*. Sinauer Associates, Sunderland, Massachusetts.

Wolfe KH, Li WH, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences, USA*, **84**, 9054–9058.

Wright JW (1955) Species crossability in spruce in relation to distribution and taxonomy. *Forest Science*, **1**, 319–349.

Wu J, Krutovskii KV, Strauss SH (1998) Abundant mitochondrial genome diversity, population differentiation and convergent evolution in pines. *Genetics*, **150**, 1605–1614.

This collaborative project reflects the interests of the authors to gain a better understanding of the genetic diversity, biogeographical history, and evolutionary processes in forest trees. Juan Pablo Jaramillo-Correa is a postdoctoral fellow studying the genetic diversity and phylogeography of conifers. Jean Beaulieu is a research scientist interested in quantitative genetics and problems related to the genetic diversity of tree species. F. Thomas Ledig is a population geneticist interested in the evolutionary history and conservation genetics of trees including threatened temperate and subtropical species. Jean Bousquet is a professor involved in the application of molecular and genomic approaches to studying the evolutionary biology of plant and tree species.
