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Glacial refugia of limber pine (*Pinus flexilis* James) inferred from the population structure of mitochondrial DNA

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Abstract

To make inferences about the glacial refugia that harboured the limber pine, *Pinus flexilis* James, we examined the range-wide population structure of mitochondrial DNA (mtDNA) with eight size variants in the second intron of *nad1*. The data consisted of haplotypes from 704 trees collected from 40 localities. The value of F_{ST} for these populations was 0.80, which is a much larger value than has been reported for allozymes and chloroplast DNA (cpDNA) in limber pine, and it suggests that the number of seeds moving among localities per generation is ≈ 0.12. Gene flow of this magnitude would allow mutation and subsequent genetic drift to have a substantial impact on the population structure of mtDNA. The majority of the mtDNA haplotypes are restricted to minor portions of the geographical range. The data are consistent with mtDNA differentiation in seven glacial refugia, followed by dispersal out of those refugia.

Keywords: gene flow, geographical variation, glacial refugia, limber pine, mtDNA

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Introduction

The purpose of this study was to survey mitochondrial DNA (mtDNA) variation across the range of limber pine, *Pinus flexilis*, to test whether the pattern of geographical variation of mtDNA is consistent with the hypothesis of differentiation in isolated glacial refugia and subsequent dispersal. This hypothesis was suggested by results of our previous survey of mtDNA, chloroplast DNA (cpDNA), randomly amplified polymorphic DNA (RAPD) and allozymes among seven populations along an elevational transect in the Front Range of Colorado (Latta & Mitton 1997). Among-population variation was low for 10 allozyme loci (mean $F_{ST} = 0.016$) and for one cpDNA marker ($F_{ST} = 0.013$), and all of these estimates were homogeneous. However, both the mtDNA ($F_{ST} = 0.679$) and the RAPD markers (predominantly, if not exclusively, nuclear) had much higher levels of population differentiation, and the RAPD markers gave heterogeneous estimates of F_{ST}. The majority of the population differentiation was produced by a single population, which was strikingly different for mtDNA and RAPD markers, consistent with a zone of secondary contact. That study (Latta & Mitton 1997) tested and rejected the hypothesis that genetic variation was associated with an environmental gradient, but the data suggested the hypothesis that mtDNA and, to a lesser extent, RAPD markers became differentiated in refugia east of the Rocky Mountains and in the Great Basin.

Evidence from two sources reveal that limber pine resided in several, and perhaps in many, refugia when glaciers displaced the trees from the high mountains. A glimpse of the ancient distribution of limber pine can be gained by examining the macrofossils in packrat middens—materials preserved in middens can be identified and carbon dated to reconstruct plant communities of the past (Betancourt *et al*. 1990, 1991). Studies of packrat middens led Wells (Wells 1983a; Wells & Stewart 1987) to propose that the limber pines in eastern Colorado (Pawnee Buttes) and eastern Wyoming (Pine Bluffs) are relicts of the last glacial maximum, when conifers, including limber pine and Engelmann spruce, moved off the mountains and spread into present-day Kansas, Oklahoma and Texas. Similar studies revealed that limber pine was found at numerous sites between 4000 and 8000 feet in elevation in the Great Basin and on the Colorado Plateau (Wells 1983b; Betancourt 1990; Thompson 1990). In addition, macrofossils

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document the presence of limber pine 22 720 years before present (BP) in the mountains of Colorado (Fremont Co.) and between 40 000 and 27 000 years BP in Bighorn Co., Wyoming, less than 300 km south of the southernmost margin of the glacier (Wells & Stewart 1987). Thus, it appears that limber pine may have had at least four glacial refugia, but it is probable that there were other refugia that we have not yet identified.

Genetic markers in the mtDNA of pines are ideal for detecting ancient events of differentiation and dispersal (Sinclair *et al*. 1999). The nuclear, chloroplast and mitochondrial components of the genomes of most conifers have different modes of inheritance, disparate potentials for gene flow and heterogeneous degrees of population structure (Mitton 1997). For most species in the family Pinaceae, mtDNA is inherited maternally (Neale *et al*. 1991; Marshall & Neale 1992; Dong & Wagner 1994; but see Wagner *et al*. 1991; Neale *et al*. 1989, 1991), and is dispersed only by the movement of seeds, whereas cpDNA is inherited paternally, and is dispersed first by wind-borne pollen and then by the movement of fertilized seeds (Neale *et al*. 1986; Strauss *et al*. 1989; Dong & Wagner 1994). Nuclear genes, like cpDNA, are dispersed by both pollen and seeds. In the absence of selection, gene flow is inversely related

Table 1 Mitochondrial DNA (mtDNA) haplotype frequencies in limber pine

Region	Locality	1	2	3	4	5	6	7	8
Southern Rockies	1 Sandia Crest, NM	15		8					
	2 Red River, NM			16					
	3 Magdalena Mts, NM	15							
	4 Manzanos Mts, NM			6					
	5 Gallinas Mts, NM			2					
	6 Mt. Withington, NM	4		4					
	7 Sierra Blanca, NM			6					
	8 Sacramento Mts, NM			1					
	9 Sangre de Cristo, NM			2					
	10 Black Mt, CO			10				5	
	11 Independence Pass, CO							10	
	12 Monarch Pass, CO							12	
	13 Trout Creek Pass, CO							2	
	14 Sheep Mt, CO			4				38	
	15 Weston Pass, CO							20	
	16 La Veta Pass, CO			14				20	
	17 Berthoud Pass, CO	1		6					
	18 Golden Canyon, CO			5					
	19 Red Hill Pass, CO			23					
	20 Rollins Pass, CO	1		3					
	21 Niwot Ridge, CO	2		37					
	22 Hallet Peak, CO	4		36					
	23 Sugarloaf Mt, CO	2		35					
	24 Green Mt, CO	3		38					
	25 Ouray, CO					16			
	26 Durango, CO			2					
	27 Wolf Creek Pass, CO			2				6	
	28 Pawnee Buttes, CO			41					
	29 Pine Bluff, WY			37					
	30 Tie Siding, WY			8					
Great Basin	31 San Francisco Peak, AZ					10			
	32 East Humboldt Mts, NV							18	
	33 Wheeler Peak, NV					15		1	
	34 Sheep Creek, UT			11			3		
	35 Ferron Canyon, UT	1		16			3		
	36 Avintaquin, UT			18			2		
Canada	37 Whirlpool Pt. AB								28
	38 Porcupine Hills, AB			20					
California	39 Mt. Pinos, CA		20						
	40 Horseshoe Meadows, CA		13		3				

AB, Alberta; AZ, Arizona; CA, California; CO, Colorado; NM, New Mexico; NV, Nevada; UT, Utah; WY, Wyoming.

to the degree of population differentiation as measured using F_{ST}, a standardized measure of the variance of allelic frequencies. Because wind-borne pine pollen typically moves much further than pine seeds, the potential for gene flow is high for cpDNA and nuclear genes, but low for mtDNA. Empirical studies of conifers are consistent with these expectations in that the F_{ST} values of maternally inherited markers are greater than the F_{ST} values for nuclear or cpDNA markers in lodgepole pine, *P. contorta* (Wheeler & Guries 1982; Dong & Wagner 1993, 1994), Monterey pine, *P. radiata*, Bishop pine, *P. muricata*, and knobcone pine, *P. attenuata* (Hong *et al*. 1993; Strauss *et al*. 1993; Wu *et al*. 1998), and limber pine, *P. flexilis* (Latta & Mitton 1997). For example, in Bishop pine, *P. muricata*, the value of F_{ST} for allozymes is 0.22 (Millar *et al*. 1988) but the value of F_{ST} for mtDNA is 0.96 (Strauss *et al*. 1993).

Here we present a study of the population structure of mtDNA in limber pine, which was based on 704 trees sampled from 40 populations. The data suggest that the mtDNA of limber pine became differentiated in a minimum of seven glacial refugia.

Materials and methods

Sample sites are listed in Table 1 and illustrated in Fig. 1. The sites include those sampled previously by Latta & Mitton (1997) and additional sites for this study. A total of 704 trees were sampled from 40 sample sites. More detailed information on the sites is available from the corresponding author (J. B. M.).

Samples consisted of a single twig and a few needles taken from a mature tree in the field. The twigs were kept

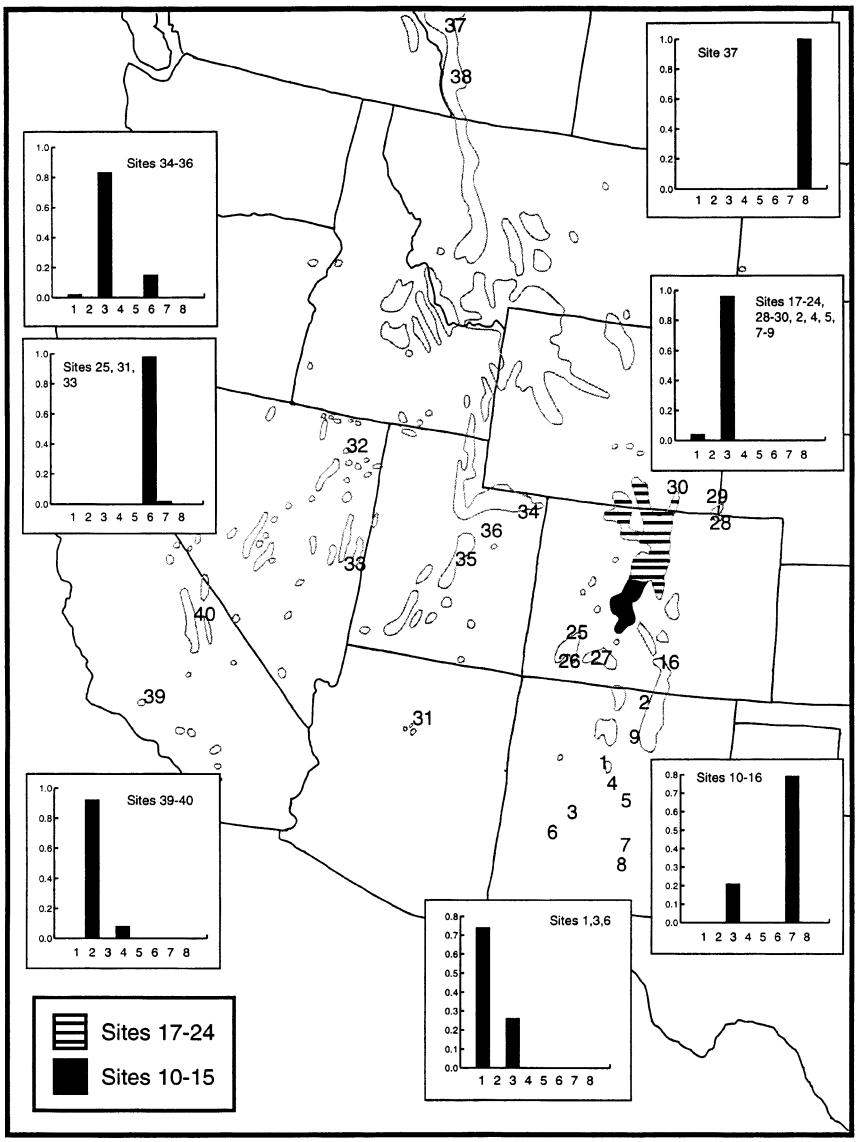

Fig. 1 The distribution of limber pine, *Pinus flexilis*, showing sample sites and the frequencies of mitochondrial DNA (mtDNA) haplotypes in selected sample sites. The site numbers correspond to those in Table 1 and Fig. 2. The full data set for mtDNA is presented in Table 1.

cool until DNA was extracted from them at the genetics facility at the University of Colorado.

Total genomic DNA was extracted from needle tissue using the hexadecyltrimethyl ammonium bromide (CTAB) extraction procedure of Doyle & Doyle (1987). The primers of Demesure *et al*. (1995) were used to amplify the second intron of *nad1* from the mitochondrial genome. The polymerase chain reaction (PCR) contained, in a 15-μL reaction volume, 50 mM KCl, 10 mM Tris-HCl, 0.01% gelatin, 3 mM $MgCl_2$, 200 μM of each dNTP, 0.5 U of *Taq* polymerase, 200 nM of each primer and 20 ng of template DNA. The amplification cycle consisted of an initial 3-min denaturation at 94 °C followed by 45 cycles of: 1 min denaturation at 94 °C, 1 min annealing and 2.5 min extension at 72 °C, with a final 10-min extension at 72 °C. The annealing temperatures employed a touchdown temperature profile, with four cycles at each of a descending series of temperatures (63 °C, 61 °C, 59 °C and 57 °C); the following 39 cycles used an annealing temperature of 55 °C. The 2.2-kb fragment was digested with *Rsa*I, and visualized by silver staining (Klinkicht & Tautz 1992) after electrophoresis on a 5% denaturing acrylamide gel. The cleaved fragments of the amplified DNA were assigned numbers: 1 was assigned to the smallest fragment and other fragments were labelled sequentially with increasing size.

Frequencies of mtDNA haplotypes were treated as allelic frequencies at a single locus for statistical purposes. F_{ST} was used to describe the variation among populations and was calculated using BIOSYS (Swofford & Selander 1981). The homogeneity of haplotype frequencies was tested using a row-by-columns test of independence, which employs χ^2. Because some of the sample sites had small sample sizes, we grouped the samples into the geographical regions listed in Table 1 for the calculation of χ^2.

Results

Eight size variants of the cleaved fragment of the second intron of *nad1* were detected (Table 1) and we refer to these as haplotypes 1 to 8. The sizes of the fragments from haplotypes 1, 3, 6, 7 and 8 formed a regular series; the sizes of these fragments were 1080, 1120, 1160, 1200 and 1240, respectively. However, the fragments from haplotypes 2, 4 and 5, which were ≈ 1100, 1130 and 1150, respectively, are interspersed in this series, disrupting the pattern of even spacing.

A row-by-columns test of independence revealed that the allelic frequencies were heterogeneous among the geographical areas ($\chi^2 = 181.1$, $P < 0.001$). The value of F_{ST} for the haplotypes was 0.80, indicating that relatively little of the variation resided within populations, and that most of the variation was among populations.

Most of the haplotypes were restricted to a small area of the range of limber pine (Table 1, Fig. 1). Haplotype 1 reached its highest frequencies in the mountains of New Mexico, where it was the most common haplotype on Sandia Crest, and was fixed in the Magdalena Mountains. Haplotype 1 was found only as a rare variant in a few of the collection sites in the Rocky Mountains in Colorado and in the Wasatch Mountains of Utah. Haplotype 2 was found only in the two sites in California; it appeared to be fixed on Mt. Pinos, and was the most common haplotype at Horseshoe Meadows. Haplotype 3 was the most widespread; it was distributed from the Sacramento Mountains of southern New Mexico to the Porcupine Hills of Alberta, and from Pawnee Buttes in eastern Colorado to the Wasatch Mountains of Utah. Haplotype 4 was only found at Horseshoe Meadows, California, where it was less common than haplotype 2. Haplotype 5 was restricted to the southwest. It was fixed at Ouray, Colorado, and in the San Francisco Peaks, Arizona, and it was nearly fixed at Wheeler Peak, Nevada. Haplotype 6 was limited to the Wasatch and Uintah Mountains of Utah, and in those areas it was less common than haplotype 3. Haplotype 7 was most common at high elevation sites along the Continental Divide in Colorado, and it was also found on Black Mountain and La Veta Pass, which are also relatively high sites. This haplotype was not detected in Utah, but it appeared again in the East Humboldt Mountains and as a rare variant on Wheeler Peak. In general, this haplotype was only detected at collection sites with elevations exceeding 3000 m. Haplotype 8 was found in just one collection site, our most northern site, at Whirlpool Point, Alberta, where it appeared to be fixed.

Discussion

It is probable that the mtDNA variation among extant populations (Table 1) evolved when glaciers displaced limber pines, restricting them to refugia that were reproductively isolated by the distances among them. Macrofossils from packrat middens and palynological data provide evidence that limber pine resided in a minimum of four glacial refugia: east of the Rocky Mountains in Colorado and Kansas (Wells 1983a; Wells & Stewart 1987) and at numerous sites in the Great Basin (Wells 1983b; Betancourt 1990; Thompson 1990), Fremont Co., Colorado (Wells & Stewart 1987) and Bighorn Co., Wyoming (Wells & Stewart 1987). Furthermore, it is probable that additional refugia existed that have not yet been discovered.

The distributions of the haplotypes are informative and suggest areas that may have served as glacial refugia and the subsequent routes of dispersal out of them (Fig. 2). For example, haplotype 1 is common in the Magdalena Mountains and on Sandia Crest in New Mexico, but rare

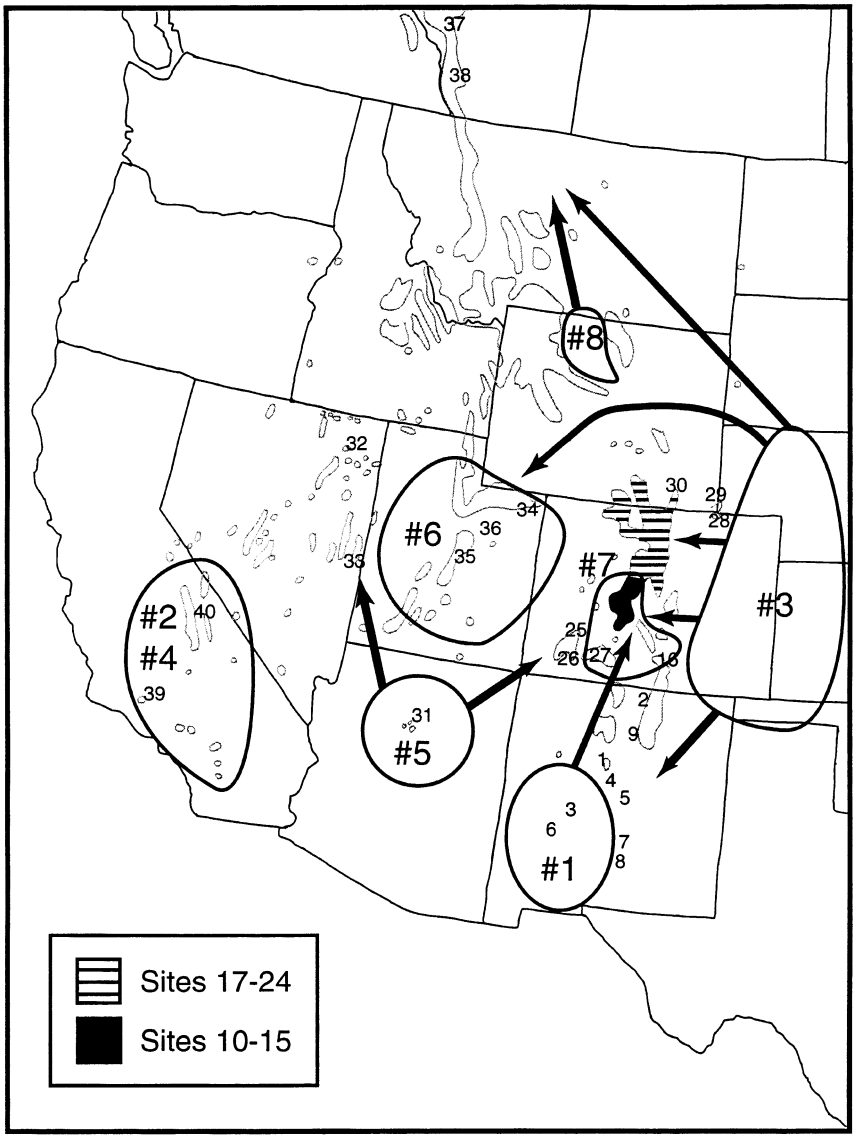

Fig. 2 Possible glacial refugia for limber pine, *Pinus flexilis*, and routes of dispersal from the refugia as the glaciers receded. The numbers of the sites correspond to those in Table 1 and Fig. 1. The numbers in bold are the haplotypes listed in Table 1.

in Colorado and Utah, suggesting that it emigrated from a refugium in southern New Mexico and moved with limited success to the north. Haplotypes 2 and 4, currently restricted to sites in California, might have become fixed in a refugium in the southern Sierra Nevada without ever invading the remainder of the range of limber pine. The distribution of haplotype 3 suggests that it predominated either on the eastern foothills of the Rocky Mountains or in refugia further east (Wells 1983a), where it is currently fixed. Haplotype 5 may have become fixed in a refugium near the San Francisco Peaks of Arizona, then migrated north to reach Wheeler Peak in Nevada and the San Juan of Colorado. Haplotype 6 probably became fixed in refugia in the Great Basin (Betancourt 1990), for it is restricted to that area. Haplotype 7 may have become fixed in Fremont County, Colorado, close to its present distribution, which meanders from La Veta Pass to the western fringe of South Park and the Continental Divide in central Colorado. Haplotype 8 is fixed in and restricted to our most northern site in Canada; perhaps this haplotype became fixed in the refugium in Bighorn County, Wyoming (Wells & Stewart 1987), less than 300 km south of the glacier's margin when it reached its maximum. Trees from this refugium probably spread to the north as the Wisconsin glacier receded.

Dispersal out of the glacial refugia would have been assisted by Clark's nutcracker, *Nucifraga columbiana*, which harvest and cache the seeds of limber pine (Tomback & Linhart 1990). Although the birds would have helped to carry the seeds for expanding populations, their flight distances are insufficient to maintain gene flow of mtDNA across today's fragmented distribution. Flight distances of a few hundred metres are probably typical, although

flights up to 22 km have been reported (VanderWall & Balda 1977; Tomback & Linhart 1990). While 22 km is a substantial distance, this range of dispersal is insufficient to connect all the extant portions of the range of limber pine (Fig. 1). For example, we estimate the minimal distance between populations of limber pine in the Rocky Mountains of Colorado and the Uintah Mountains of Utah to be 150 km. This gap could be crossed by wind-borne pollen, but not by Clark's nutcrackers carrying seeds. Similarly, limber pine on Mt. Pinos in California and in the Magdalena Mountains may be exchanging pollen (and therefore nuclear genes and cpDNA) with pines in other populations, but the mtDNA in these populations may be perfectly isolated. Gene flow of nuclear and chloroplast genes is substantial in limber pine (Latta & Mitton 1997; Schuster *et al*. 1989), as it is in most conifers. For example, when 10 polymorphic allozyme loci were used to survey population structure for limber pine populations in the Front Range of Colorado, the mean F_{ST} was 0.02, and inferences of the number of migrants among populations ranged from five to 250 (Latta & Mitton 1997). Similarly, when 23 polymorphic allozyme loci were used to survey limber pine populations in the Great Basin and Colorado Plateau, the mean F_{ST} was 0.101 (Hamrick *et al*. 1994), yielding an estimate of 2.2 for the number of migrants among populations per generation. In contrast, the F_{ST} for mtDNA for the limber pine populations in the Front Range was 0.67 (Latta & Mitton 1997), yielding an estimate of migrants per generation of 0.24. The value of F_{ST} for the data in Table 1 is 0.80, from which we infer that the average number of migrants per generation is 0.12. Thus, in comparison to the genes moved by pollen (nuclear genes and cpDNA), the genes moved only by seeds (mtDNA) have much lower rates of gene flow.

Not only does the limited gene flow of mtDNA probably account for the substantial geographical structuring, but it also provides an explanation for the sharp discontinuity in mtDNA haplotypes found in Colorado. The populations on the fringe of South Park (Black Mountain, Red Hill Pass) and the populations to the east have predominantly haplotype 3, while all populations to the west of South Park (Sheep Mountain, Monarch Pass, Independence Pass, Weston Pass) are fixed or nearly fixed for haplotype 7. For example, most of the limber pine on Sheep Mountain have haplotype 7, but Red Hill Pass, less than 16 km east of Sheep Mountain, is fixed for haplotype 3. Thus, the mtDNA data indicate that there is little or no exchange of seed between these localities. This abrupt discontinuity might be an area of secondary contact of limber pines from genetically differentiated refugia. The limited dispersal of seeds would allow mtDNA to record secondary zones of contact long after gene flow had erased similar differentiation for nuclear and chloroplast genes.

We cannot determine whether the haplotype diagnostic for a geographical area is a unique mutation that became fixed, or is the sole haplotype remaining after lineage sorting. At one extreme is the hypothesis that the preglaciation limber pines had little or no mtDNA variation, and that all of the haplotypes detected here arose by new mutations and became fixed in refugia isolated by distance. At the other extreme is the hypothesis that the preglaciation limber pines had abundant mtDNA variation, and that lineage sorting fixed different haplotypes in the various refugia. Understanding the phylogenetic relationships among the haplotypes might allow us to reject one of the hypotheses, and to refine the hypotheses concerning the number of refugia, their locations and routes of emigration from them. For example, if the haplotypes present within a region were sister lineages, then they probably represent *de novo* mutation, whereas if they were more distantly related, lineage sorting is the more appropriate explanation. Currently, however, owing to the extreme conservation of plant mtDNA sequences, we lack the variable sites needed to estimate phylogenetic relationships.

Because the haplotypes are identified solely by size, it is possible that some of our haplotype designations contain homoplasies; haplotypes identical in size may not be identical by descent. For example, we found haplotype 7 along the Continental Divide in Colorado and also in the East Humboldt Mountains of Nevada. Are these haplotypes identical by descent or are they the product of separate mutational events? This a problem common to studies of organellar genomes, for even haplotypes homogeneous for a particular sequence may be heterogeneous for other genes in the organellar genome. However, despite the potential for additional variation within the haplotypes we have identified, the strong geographical structuring of size variants clearly indicates that seed exchange between glacial refugia has been very limited.

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Jeff Mitton is an evolutionary geneticist whose research on plants and animals focuses on the forces that influence population structure. Brian Kreiser recently finished his doctoral theses research on the phylogeography of the plains killifish, *Fundulus zebrinus*, and he is currently working on several projects in conservation genetics of fishes. Bob Latta is a molecular plant population geneticist, and he recently finished his doctoral thesis working on the population structure of limber pine, as revealed by mtDNA, cpDNA, allozymes and RAPDs. Both B. Kreiser and R. Latta received their doctoral degrees at the University of Colorado, working in J. Mitton's laboratory.
