Chloroplast DNA Diversity Among Trees, Populations and Species in the California Closed-Cone Pines (*Pinus radiata*, *Pinus muricata* and *Pinus attenuata*)

Yong-Pyo Hong, Valerie D. Hipkins and Steven H. Strauss¹

Department of Forest Science, FSL 020, Oregon State University, Corvallis, Oregon 97331-7501

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ABSTRACT

The amount, distribution and mutational nature of chloroplast DNA polymorphisms were studied via analysis of restriction fragment length polymorphisms in three closely related species of conifers, the California closed-cone pines—knobcone pine: *Pinus attenuata* Lemm.; bishop pine: *Pinus muricata* D. Don; and Monterey pine: *Pinus radiata* D. Don. Genomic DNA from 384 trees representing 19 populations were digested with 9–20 restriction enzymes and probed with cloned cpDNA fragments from Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] that comprise 82% of the chloroplast genome. Up to 313 restriction sites were surveyed, and 25 of these were observed to be polymorphic among or within species. Differences among species accounted for the majority of genetic (haplotypic) diversity observed [$G_{st} = 84(\pm 13)\%$]; nucleotide diversity among species was estimated to be $0.3(\pm 0.1)\%$. Knobcone pine and Monterey pine displayed almost no genetic variation within or among populations. Bishop pine also showed little variability within populations, but did display strong population differences [$G_{st} = 87(\pm 8)\%$] that were a result of three distinct geographic groups. Mean nucleotide diversity within populations was $0.003(\pm 0.002)\%$; intrapopulation polymorphisms were found in only five populations. This pattern of genetic variation contrasts strongly with findings from study of nuclear genes (allozymes) in the group, where most genetic diversity resides within populations rather than among populations or species. Regions of the genome subject to frequent length mutations were identified; estimates of subdivision based on length variant frequencies in one region differed strikingly from those based on site mutations or allozymes. Two trees were identified with a major chloroplast DNA inversion that closely resembled one documented between *Pinus* and *Pseudotsuga*.

As a consequence of its slow rate of evolution and low polymorphism, there have been few efforts to quantify the nature and distribution of chloroplast DNA (cpDNA) variability within populations and species (reviewed by SOLTIS, SOLTIS and MILLIGAN 1992). Organelle genomes may have substantially different patterns of genetic diversity than nuclear genes (BIRKY 1988) because of their low mutation rates (WOLFE, LI and SHARP 1987), lower effective population sizes and the potential for periodic selection (MARUYAMA and BIRKY 1991). MILLIGAN (1991) made calculations based on work in *Hordeum* (NEALE *et al.* 1988) and *Lupinus* (BANKS and BIRKY 1985) that suggested substantial subdivision for cpDNA variants among populations ($F_{st} > 70\%$). Studies of *Tolmiea* (SOLTIS *et al.* 1989), *Tellima* (SOLTIS *et al.* 1991) and *Datisca* (LISTON, RIESEBERG and HANSON 1992) reported strong differentiation among limited population samples. Evidence from phylogenetic studies suggests that fixation of foreign cpDNA genomes resulting from hybridization can sometimes occur rapidly, and without significant changes of nuclear gene frequencies (reviewed by RIESEBERG and SOLTIS 1991; and by SCHAAL, O'KANE and ROGSTAD 1991).

Most population genetic studies of cpDNA have focused on complex restriction fragment length polymorphisms that may result from length mutations or rearrangements (SOLTIS, SOLTIS and MILLIGAN 1992). Such polymorphisms are often associated with localized hotspots that contain repetitive DNA (PALMER 1985; HIPKINS 1993). Relative to the genome as a whole, their rates of mutation are therefore likely to be variable and mutant alleles subject to reversion and homoplasia (*e.g.*, KIM, TURNER and JANSEN 1992). Because of their mutational complexity and lack of representativeness of the genome, they provide biased estimates of nucleotide diversity and thus may also give rise to incorrect estimates of genetic subdivision. Restriction site polymorphism is therefore preferred for assessing population genetic processes.

The goal of this study was to examine the nature and distribution of chloroplast DNA genetic diversity in a group of species that had already been well characterized by several other means, including allozymes, terpene composition, crossability, morphology and paleohistory (reviewed by MILLAR 1986). Monterey and bishop pine are maritime species that occur in a limited number of disjunct, largely allopatric populations; knobcone pine is a montane species with

¹ To whom reprint requests should be sent.

FIGURE 1.—Distribution of Monterey (*P. radiata*), knobcone (*P. attenuata*) and bishop pines (*P. muricata*) (darkened areas); population labels show the locations of the sampled populations.

a wider range but also with many discontinuities in its distribution (Figure 1). Because of the close relationships among the species—and the infraspecific taxonomic designations given to several of the distinct populations—it provides an opportunity to observe the distribution of cpDNA diversity in a group of woody plants that has relatively recently undergone speciation and population isolation. We report that (1) although levels of within-population restriction site polymorphism are extremely low, strong genetic differentiation may rapidly develop among populations and species compared with nuclear genes; (2) the distribution of restriction site polymorphism can be very different from that of length polymorphism; and (3) major genome inversions can exist within populations and appear similar (convergent) to that observed among genera.

MATERIALS AND METHODS

Plant materials: We sampled a total of 384 trees originating from 19 populations (Figure 1, Table 1). All samples of knobcone pine were from trees growing in natural populations. The Klamath population was sampled over a 2-mile transect adjacent to U.S. Interstate 5 north of Shasta Lake Recreation Area, California (latitude 40°50', longitude 122°45'). The Sierra Nevada population was sampled over an 2-mile transect located adjacent to U.S. Interstate 80 near Auburn, California (latitude 38°54', longitude 121°08'). The Oakland population was sampled over an 1.5-mile transect along Flicker Ridge, in the hills east of Oakland, California (latitude 37°50', longitude 122°30'). The Santa Ana population was sampled from widely spaced trees in the Santa Ana mountains near Corona, California (latitude 33°50', longitude 117°37').

Bishop and Monterey pines were sampled from gene conservation and genetic test plantations in three locations: Albany, California (Gill Tract) and LaFayette, California (Russell Reservation) established by W. J. LIBBY and associates of the University of California at Berkeley; and the U.S. Naval Weapons Research Station in Concord, California, established by the U.S. Forest Service, Pacific Southwest Experiment Station in Berkeley, California (maintained by F. T. LEDIG and associates). All of the trees in these plan-

TABLE 1
Sample origins and sizes

Species/Population	No. of Trees	Abbreviation	Origin ^a
<i>P. radiata</i>			
Año Nuevo	30	MANO	G
Cambria	28	MCAM	G
Cedros Island	27	MCED	G & R
Guadalupe Island	24	MGUA	G & R
Monterey	27	MMOT	G
Total	136		
<i>P. attenuata</i>			
Klamath	25	KKLA	NP
Oakland	25	KOAK	NP
Sierra	25	KSIE	NP
Santa Ana	25	KSAA	NP
Total	100		
<i>P. muricata</i>			
Mendocino	6	BMEM	C & R
Trinidad	7	BTRI	R
Marin	5	BMAR	C & R
Sonoma	7	BMSO	R
Monterey	12	BMON	C & R
San Luis Obispo	12	BSAL	C & R
San Vicente	20	BSAV	C & R
Santa Barbara	19	BASB	C & R
Santa Rosa	17	BSAR	C & R
Santa Cruz	43	BSAC	C & R
Total	148		
Total Sample	384		

^a Symbols for origins. Experimental plantations: C = Concord; G = Gill Tract; R = Russell Reservation. NP = natural population. See text for details.

tations were either clonal replicates of trees from natural populations or derived from seeds collected in natural populations.

DNA analysis: Total cellular DNA was extracted from 40 g of sampled needles as described by WAGNER *et al.* (1987). Modifications to this protocol, and methods for restriction enzyme digests, electrophoresis, blotting, probe labeling, hybridization and washing are described in STRAUSS, HONG and HIPKINS (1993) and in STRAUSS and DOERKSEN (1990). To reveal cpDNA polymorphisms blots

FIGURE 2.—Linearized Douglas-fir cpDNA restriction site map for *KpnI*, *SstI* and *XbaI*. Maps and clones were developed by STRAUSS *et al.* (1988), Tsai and STRAUSS (1989), HIPKINS (1993) and ourselves. Dashed lines within probe sets indicate uncloned cpDNA fragments (thus, not included as hybridization probes). Arrow heads with asterisk denote approximate inversion borders in Douglas-fir relative to Monterey pine (STRAUSS *et al.* 1988). Probe sets were: Set A = *XbaI*-6.4, 8.9, 4.8 and *KpnI*-1.2, 1.6, 2.6, 6.0, 0.7. Set B = *XbaI*-11.2, 2.5, 3.8, 1.6 and *KpnI*-8.7. Set C = *XbaI*-5.9b, 6.0, 2.7, 3.2a and *KpnI*-6.5. Set D = *XbaI*-5.1, 1.9, 0.8 and *SstI*-10.8a, 2.6, 6.7, 4.8

were hybridized with four mixtures of equal amounts of cloned Douglas-fir cpDNA fragments (Figure 2) that together cover 82% of the chloroplast genome.

Strategy for surveying polymorphisms: We used a two-stage procedure to detect cpDNA polymorphisms. For the preliminary screen, we randomly chose two trees from each of the 19 sampled populations, plus a single tree of *Pinus oocarpa* Scheide [University of California Botanical Garden, Berkeley, California, accession no. 67.164; native in Valle de Bravo, Mexico (latitude 13°14', longitude 100°07')]. We then digested these DNAs with 17 restriction enzymes with 6-bp recognition sites (*Bam*HI, *Bcl*II, *Bgl*I, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sst*I, *Stu*I, *Xba*I, *Xho*I and *Xmn*I) and three enzymes with 4-bp recognition sites (*Hin*fI, *Hpa*II and *Msp*I), and probed their blotted DNA with our cpDNA probe sets. Only those nine enzymes (*Bam*HI, *Bcl*I, *Dra*I, *Eco*RI, *Eco*RV, *Kpn*I, *Xba*I, *Xho*I and *Xmn*I) that detected polymorphisms either within or between species in this primary survey were retained for the full survey of all 384 study trees.

Quantitative analyses: Haplotypes were recognized on the basis of having unique restriction fragment patterns over the various restriction enzyme and probe combinations studied. Only clearly interpretable site mutations were considered in nucleotide diversity analyses; genome polymorphisms and length mutations were excluded. To avoid counting fragments and site mutations more than once, for each enzyme identical blots were hybridized with each probe set and autoradiograms from adjacent probe sets carefully compared. The number of unique fragments observed was assumed equal to the total number of sites surveyed. Because of the gaps in genome coverage provided by our probe sets, however, this probably gives a slight underestimate of the number of sites surveyed. The number of different kinds of haplotypes in each population was used to estimate nucleotide diversity and its standard errors among and within populations and species (with the computer program HAPLO: Lynch and Crease 1990).

The relative degree of differentiation at each hierarchical level studied was quantified by three parameters: N_{st} , G'_{st} and F'_{st} . The parameter N_{st} weights evolutionary distances among pairs of haplotypes based on sequence divergence estimated by maximum likelihood (NEI and TAJIMA 1983). It is calculated as $N_{st} = V_b / (V_w + V_b)$, in which V_w is the average number of substitutions per site for random pairs of chloroplast DNA within a hierarchical level (*e.g.*, within populations), and V_b is the average number of substitutions

per site for random pairs of chloroplast DNA among hierarchical levels (*e.g.*, among populations) (LYNCH and CREASE 1990). For V_b , V_w and N_{st} calculations, the 11 enzymes that were used only in the preliminary survey were assumed to be monomorphic in all trees. This gives a more accurate estimate of gene diversity than the inflated value that would be obtained if only the nine polymorphic enzymes of the 20 studied were used. Results from the nine-enzyme survey showed that only a single intrapopulation polymorphism of a total of 25 were not also interpopulation or interspecies polymorphisms (data below), and thus would have been missed by our preliminary screen. Thus, our gene diversity estimates should be biased downward by only about 4% (1/25).

Nei's (1977) fractional gene diversity statistic G'_{st} was calculated using haplotype frequencies (where haplotypes are treated as alleles at a single genetic locus). The estimates of N_{st} , G'_{st} , and their standard errors were calculated by HAPLO. Wright's F -coefficient for population subdivision F'_{st} was estimated as in WEIR and COCKERHAM (1984). It, and standard deviations derived from jackknifing over populations, were calculated from haplotype frequencies with a computer program described by B. S. WEIR (1990). We also calculated G'_{st} via GENESTAT (WHITKUS 1988) and results were found to be nearly identical—and always within 2%—of results from HAPLO; GENESTAT data are therefore not presented. Hierarchical F -statistics describing haplotype subdivision in the species-group as a whole were estimated using the computer program Biosys-1 (version 1.7; Swofford 1989).

RESULTS

Preliminary screen for polymorphism: In the preliminary screen we assayed 255 restriction sites with 17 6-bp recognition enzymes, and 58 sites with three 4-bp enzymes, giving a total of 313 sites and 1762 bp assayed per tree. This is equivalent to study of 1.5% of the 120-kb pine chloroplast genome (STRAUSS *et al.* 1988). Twenty-four of the 313 sites were polymorphic either within or among species (Table 2); note that one of the 23 mutant phenotypes listed in Table 2 results from two point mutations. These 24 variable and 289 monomorphic sites could be assigned to nine different haplotypes.

TABLE 2
Restriction fragment phenotypes used to infer site mutations

O ^a B S V	E N Z Y	P R O B	M M M M M K K K K B B B B B B B B B O A C M G C K O S S M T M S M S S S S S O N A O U E L A A I E R A O O A A A A A C O M N A D A K A E N I R N N B L R V C A	P H E N
1.	<i>Bam</i> HI	A	X X X X X O O O O X X X X X X X X X NI	2.84, 2.02 + 0.62
2.	<i>Bam</i> HI	A	O O O O O X X X X O O O O O O O O O O	3.17, 3.10 + 0.07
3.	<i>Bcl</i> I	A	O O O O O O O O O X X X X X O O O O O	7.98, 7.77 + 0.21
4.	<i>Bcl</i> I	A	X X X X X X X X X O O O O O X X X X X	5.09, 4.57 + 0.52
5.	<i>Dra</i> I	A	X X X X X O O O O X X X X X X X X X NI	6.61, 6.09 + 0.52
6.	<i>Dra</i> I	A	O O O O O X X X X O O O O O O O O O NI	7.42, 4.08 + 3.34
7.	<i>Dra</i> I	A	X X X X X X X X X O O O O O ^b X X X X NI	3.34, 2.87 + 0.47 ^c
8.	<i>Eco</i> RI	B	X X X X X X X X X O O O O O O O O O X	4.42, 3.03 + 1.39
9.	<i>Eco</i> RI	B	X X X X X X X X X O O O O O X X X X X	7.71, 4.40 + 3.31
10.	<i>Eco</i> RI	B	X X X X X X X X X O O O O O X X X X X	4.73, 4.21 + 0.52
11.	<i>Eco</i> RI	B	X X X X X X X X X X X X O O O O O O ^d eX	1.93, 1.40 + 0.53 ^c
12.	<i>Eco</i> RI	C	X X X X X X X X X X X X X ^f O O O O ^g O ^h X	4.67, 3.00 + 1.67 ^c
13.	<i>Eco</i> RI	A	X ⁱ X X ^j X X O O O O X X X X X X X O X X	10.48, 8.64 + 1.84 ^c
14.	<i>Eco</i> RV	A	X X X X X O O O O X X X X X X X X X NI	5.15, 3.37 + 1.78
15.	<i>Eco</i> RV	A	X X X X X X X X X O O O O O X X X X NI	5.15, 4.70 + 0.45
16.	<i>Eco</i> RV	A	X X X X X X X X X O O X X X X X X X NI	4.25, NO
17.	<i>Kpn</i> I	A	X X X X X O O O O X X X X X X X X X X	22.10, 19.8 + 2.3
18.	<i>Xba</i> I	A	O O O O O X X X X O O O O O O O O O O	19.22, 11.24 + 6.19 + 1.79 ^k
19.	<i>Xba</i> I	A	O O O O O X X X X O O O O O O O O O X	5.19, 4.68 + 0.51
20.	<i>Xho</i> I	D	O O O O O X X X X O O O O O X X X X NI	5.52, 4.25 + 1.27
21.	<i>Xmn</i> I	A	X X X X X X X X X O O X X X X X X X NI	8.25, 6.65 + 1.60
22.	<i>Xmn</i> I	A	X X X X X X X X X O O O O O X X X X NI	8.25, 7.60 + 0.65
23.	<i>Xmn</i> I	A	O O O O O X X X X O O O O O O O O O O	2.79, 2.70 + 0.70

^a OBSV, observation number; ENZY, restriction enzyme; PROB, probe-set number; PHEN, fragment phenotype observed and used to infer mutation (kb); O, restriction site present; X, restriction site absent; NI, presence/absence of point mutation not interpretable; NO, fragments not observed; OOCA, *Pinus oocarpa*; other species abbreviations given in Table 1.

^b Uncommon phenotype identical to BSAB; see footnote c.

^c Polymorphism observed within some populations; most common phenotype is shown in table and uncommon phenotypes are indicated by footnotes c and d-j.

^{d,e} Uncommon phenotype identical to MANO.

^f Uncommon phenotype identical to BSAB.

^{g,h} Uncommon phenotype identical to MANO.

^{i,j} Uncommon phenotype was loss of 9.9-kb band and replacement with 7.2 + 2.7-kb bands (Figure 3).

^k Phenotype was result of two adjacent point mutations.

Nucleotide diversity within populations: Twenty of the 24 polymorphic sites showed no variability within our population samples. Of the 19 populations surveyed, five contained polymorphisms—two populations of Monterey pine [Año Nuevo (Figure 3) and Monterey] and three of bishop pine (San Vicente, Santa Cruz and Monterey) (Table 2). Estimated nucleotide diversities within the five populations were all low—0.002, 0.002, 0.011, 0.003 and 0.033%; observed haplotype frequencies were 0.033, 0.037, 0.100, 0.023 and 0.500, respectively. Only one additional kind of polymorphism was discovered in the full survey that had not been detected in the preliminary screen; it was the one present at low frequencies in the Año Nuevo and Monterey populations of Monterey pine (Table 2: footnotes i and j). All 100 individuals from the four geographically distant populations of knobcone pine were monomorphic. Mean nucleotide diversities within populations were 0.005% or below for bishop pine and Monterey pine (Table 3).

Nucleotide diversity among populations: Nucleo-

FIGURE 3.—Autoradiogram showing examples of both cpDNA restriction site and length variation among three trees within the Año Nuevo population of Monterey pine. DNAs were digested with *Eco*RI and hybridized with probe set A. Sizes (kb) at right are of fragments affected by presence/absence of a site mutation; sizes at left are of the DNA size standard (*Hind*III digest of phage lambda DNA). The asterisk at left indicates the position of fragments containing small deletion/insertion mutations.

tide diversity among populations was substantially higher than that within populations. Due to a com-

TABLE 3
Estimates of subdivision based on nucleotide diversities

	V_w (%)	V_b (%)	N_{st} (%)
Pooled species ^a	0.003 (± 0.002) ^b	0.321 (± 0.076)	99.2 (± 0.6)
Species	0.042 (± 0.041)	0.307 (± 0.096)	88.0 (± 10.6)
Populations in species ^c			
<i>P. radiata</i>	0.001 (± 0.001)	-0.000 (± 0.002)	-2.2 (± 88.1)
<i>P. muricata</i>	0.005 (± 0.004)	0.223 (± 0.063)	97.9 (± 1.8)
Regional ^d	0.008 (± 0.007)	0.227 (± 0.071)	96.6 (± 2.7)
Intermediate	0.011 (± 0.011)	0.007 (± 0.015)	40.1 (± 45.9)
Southern	0.003 (± 0.002)	0.000 (± 0.003)	8.0 (± 78.4)

^a Pooled species refers to diversity among and within populations in the group without regard to species.

^b Estimates are followed by standard errors in parentheses.

^c *P. attenuata* and the Northern group of *P. muricata* were excluded from individual analyses because they showed no diversity among or within populations.

^d The regional analyses examine variation among three genetically similar groups of populations within *P. muricata*: Trinidad and Mendocino (Northern); Marin, Sonoma, and Monterey (Intermediate); and the remaining five populations south of Monterey (Southern). Intermediate and southern analyses examine variation among populations within these regions.

plete lack of detected site polymorphism, no population differentiation was observed in knobcone pine. In Monterey pine, due to a very low level of polymorphism within populations and a trivial amount of haplotype frequency differentiation among populations, nucleotide diversity among populations was estimated to be zero (*i.e.*, a very low negative estimate). In contrast, nucleotide diversity among populations in bishop pine was significantly greater than zero, and population subdivision was substantial (N_{st} , G'_{st} and F'_{st} all greater than 80%). This high degree of subdivision resulted from both strong, fixed differences in haplotype frequencies among population samples ($V_b = 0.223\%$) as well as a paucity of diversity within populations ($V_w = 0.005\%$) (Tables 3 and 4). When haplotype frequencies for the entire species-group were analyzed as a single hierarchical unit with Biosys-1 (SWOFFORD 1989), F' was 88.7% for populations relative to species, 48.8% for species relative to the total group and 94.2% for populations relative to the total group.

Three regional groups of bishop pine populations accounted for the large majority of its population subdivision: a northern region composed of Trinidad and Mendocino populations; an intermediate region composed of Marin, Sonoma and Monterey populations; and a southern region composed of all five populations south of Monterey. Apart from minor polymorphisms, trees within these regions appeared to be fixed for site mutations that differentiated them from trees in other regions. The northern region differed from the intermediate region in two site mutations, and from the southern region by 11 site mutations; the intermediate and southern region differed by nine mutations (Table 5).

Estimates of subdivision among bishop pine regions were large, and virtually identical to that among populations. When the species was subdivided into its three regional groups and analyzed separately, its high

degree of population subdivision disappeared for the northern (monomorphic) and southern groups, but remained moderately high for the intermediate group (N_{st} , G'_{st} and F'_{st} all above 23%) (Tables 3 and 4).

Nucleotide diversity among species: Nucleotide diversity among species ranged from 0.11–0.66% (Table 5), far exceeding that among populations within species except in the case of bishop pine, where it was only marginally greater (V_b of 0.31% for species *vs.* 0.22% for populations of bishop pine: Table 3). Nucleotide diversities indicated that knobcone pine was most divergent from the other species, differing by an average of 0.41% from bishop and Monterey pines—which differed from one another by only 0.09%. Relative to diversity within hierarchical levels, however, population subdivision in bishop pine exceeded or was equal to that among species; for example, N_{st} was 88.0% among species but 97.9% among bishop pine populations (Table 3 and 4).

Genomic diversity: One rearrangement was found in two individuals in the southernmost population of bishop pine from San Vicente, Mexico. The rearrangement appeared to result from an inversion because hybridization with probe sets B and D gave two new pairs of bands that could not have been the result of simple site mutations, and each of whose sizes were equal when summed ($8.2 + 6.9 = 15.1$, and $6.2 + 8.9 = 15.1$ kb) (Figure 4). Hybridization with specific Douglas-fir cpDNA clones (*Sst*I 6.7 and 4.8, and *Xba*I 2.5 and 3.8; Figure 2) further narrowed the location of the inversion borders, which appear to be identical to a nearly 50-kb inversion that differentiates Monterey pine from Douglas-fir (STRAUSS *et al.* 1988).

At least three different parts of the genome were subject to length mutations. Hybridizations with probe-set C revealed two mutable regions, and probe-set A revealed one mutable region. Due to small changes in fragment sizes (<100 bp), however, we were unable to confidently sort and recognize variants

TABLE 4
Estimates of population subdivision based on haplotype frequencies

	H' _s (%) ^a	D' _{st} (%) ^b	G' _{st} (%) ^c	F' _{st} (%) ^d
Pooled species	5.5 (±3.4)	78.0 (±3.4)	93.4 (±3.2)	93.4 (±2.8)
Species	15.9 (±0.0)	84.1 (±1.8)	84.1 (±13.2)	81.9 (±15.6)
Populations in species				
<i>P. radiata</i>	2.8 (±0.0)	−0.0 (ud) ^e	−0.9 (ud)	−1.1 (±1.1)
<i>P. muricata</i>	9.0 (±6.5)	63.2 (±23.9)	87.5 (±8.0)	81.6 (±10.0)
Regional ^f	15.8 (±12.7)	84.2 (±11.4)	84.2 (±10.8)	88.7 (±12.4)
Intermediate	22.2 (±0.0)	11.1 (±3.8)	33.3 (±4.0)	23.9 (ud)
Southern	4.7 (±2.8)	0.2 (ud)	3.4 (ud)	1.7 (±4.7)

^a H'_s = Haplotype diversity within populations expressed as percentage; standard errors for this and other parameters in parentheses.

^b D'_{st} = Haplotype diversity among populations (H'_s − H'_s).

^c G'_{st} = Percentage of haplotype diversity among populations (100 × D_{st}/H_i).

^d F'_{st} = Subdivision estimate of Weir and Cockerham (1984) ± jackknife-derived standard deviation.

^e ud = Undefined.

^f Regional groups defined in legend of Table 3.

TABLE 5
Nucleotide diversity (%) within and among species and regional groups of populations

	<i>P. radiata</i> ^a	<i>P. attenuata</i>	<i>P. muricata</i>		
			N ^b	I	S
1.	0.00 ^c	0.39	0.27	0.25	0.11
2.	13	0.00	0.66	0.65	0.45
3.	9	22	0.00	0.05	0.32
4.	9	22	2	0.02	0.30
5.	4	15	11	9	0.00

^a All populations within *P. radiata* and *P. attenuata* were pooled because estimates of diversity among populations within these species were within 0.0001% of zero. All estimates of diversity among populations of these species and populations of the other species were virtually identical.

^b Populations of *P. muricata* are pooled into regional groups of like genetic composition defined in legend of Table 3: N = Northern; I = Intermediate; S = Southern.

^c Percent nucleotide diversity among species and regional groups above diagonal; that within-species (or within regional-groups) on diagonal; and the number of restriction sites differentiated among species or regional groups of the 24 polymorphic sites observed among populations below diagonal (for counting site differences, infrequent polymorphic sites were ignored).

at two of these three loci. The length variation revealed by probe-set A, however, appeared to be composed of three distinct phenotypes (5.62, 5.53 and 5.30 kb) (Figure 5) and were thus scored in all individuals in our full survey. The five populations of Monterey pine showed polymorphism within populations for all three phenotypes (Table 6). In knobcone pine, only the Santa Ana population showed polymorphism. All populations of bishop pine appeared to be fixed for the 5.62-kb fragment. Average intrapopulation gene diversities were 57.0% for Monterey pine, 11.1% for knobcone pine, 0% for bishop pine, and 17.3% for the three species combined. Values of G'_{st} were 7.3% for Monterey pine, 68.2% for knobcone pine, and 21.7% among the three species—each substantially different from the restriction site based estimates of 0%, 0%, and 84.1%, respectively.

FIGURE 4.—Autoradiograms showing evidence for a cpDNA inversion. Sizes are given for fragments affected by the inversion. (A) Genomic DNA of three individuals from the San Vicente population of bishop pine was cut with *Xho*I and probed with a mixture of *Xba*I-2.5 and *Xba*I-3.8 cloned fragments (mapped in Figure 2). (B) The same blot was probed with a mixture of *Sst*I-4.8 and *Sst*I-6.7 cloned fragments (mapped in Figure 2). Note that the sum of the sizes of the two new fragments (8.2 and 6.9 kb) is equal to the sum of the two homologous fragments (6.2 and 8.9 kb). (C) Genomic DNA from some of the same individuals in (A) was cut with *Eco*RV and probed with the same fragments as in (A). (D) The same blot as in (C) was probed with same fragments as in (B). Note fragment stoichiometry again (4.7 + 4.3 = 5.0 + 4.0).

DISCUSSION

We detected very little cpDNA restriction site polymorphism within or among species of the California closed cone pines. Mean nucleotide diversity within populations was 0.003%—which equates to trees within populations differing, on average, by less than four nucleotides per 120-kb chloroplast genome (STRAUSS *et al.* 1988). Although the phylogenetic implications of our cpDNA results will be reported elsewhere, this low restriction site diversity supports the practice of using very small samples for cpDNA phylogenetic analysis; a single tree from each of Monterey and knobcone pine, and three trees to represent the geographic races of bishop pine, would have been sufficient (HONG 1991). Thus, at least when site rather than length variation is studied, serious analysis of

FIGURE 5.—Autoradiogram showing diversity of cpDNA length mutations observed with *EcoRI* and probe set A. Genomic DNAs of 14 trees derived from the Año Nuevo population of Monterey pine are shown. Approximate sizes of the three length “alleles” we recognized are given at the right; those of the DNA size standard are given at the left.

intraspecific diversity seems unnecessary (cf. SOLTIS, SOLTIS and MILLIGAN 1992). In the current study, sampling a few trees per species selected from divergent populations would have provided a high degree of safety.

Published estimates of restriction site diversity within herbaceous angiosperm species have been low-comparable with those in the Closed Cone Pines (reviewed in SOLTIS, SOLTIS and MILLIGAN 1992). Our estimate of nucleotide diversity among populations of bishop pine, however, is relatively high. This is a consequence of the strong differentiation between the southern and north-intermediate geographic groups. Given their reproductive incompatibility and other distinct attributes (CRITCHFIELD 1967; MILLAR 1986; MILLAR and CRITCHFIELD 1988), they appear to deserve recognition as separate species. Our estimates of cpDNA nucleotide divergence among species (mean of 0.31%) are well within the range of estimates from angiosperms. DOEBLEY, VON BOTHMER and LARSON (1992) reviewed interspecific (congeneric) cpDNA diversity in angiosperms and found that the expected number of substitutions per 100 bases ranged from 0–14%, though most were below 2%.

The highly subdivided pattern of cpDNA nucleotide diversity among species, and among populations in bishop pine, contrasts with that typically seen for nuclear genes in long-lived woody species, and with that observed in a recent allozyme survey of the group. Long-lived woody species are highly outcrossing and usually maintain less than 10% of their genetic diversity among populations (HAMRICK and GODT 1990). In a recent allozyme survey of the three species, MILLAR *et al.* (1988) reported that 12–22% of the total diversity resided among populations within species. Although these values are larger than what is usually observed for wind-pollinated, long-lived perennials, they are several-fold less than our cpDNA estimates for bishop pine (>82%). Likewise, MILLAR *et al.* (1988) reported that 24% of the total diversity resided among species—roughly half of our hierar-

TABLE 6

Observed frequencies of restriction fragment variants caused by length mutations

Population	Fragment phenotypes (kb) ^a		
	5.62	5.53	5.30
<i>P. radiata</i>			
Año Nuevo	0.75	0.18	0.07
Monterey	0.46	0.25	0.29
Cambria	0.73	0.23	0.04
Guadalupe	0.40	0.45	0.15
Cedros	0.35	0.26	0.39
Mean	0.54	0.27	0.19
<i>P. attenuata</i>			
Klamath	1.00	0.00	0.00
Oakland	1.00	0.00	0.00
Santa Ana	0.30	0.00	0.70
Sierra	1.00	0.00	0.00
Mean	0.82	0.00	0.17

^a Length variants were discerned after *EcoRI* digest and hybridization with probe set A. Bishop pine was monomorphic for length variants with this enzyme-probe combination.

chical estimate of 49%. Similar results have been reported for oaks (*Quercus* species: reviewed by KREMER and PETIT 1993), where more than three-quarters of the cpDNA diversity can reside among populations within species, while the value for allozymes is less than 5%. In contrast to our results, however, they found that as a result of extensive introgression interspecific cpDNA diversity was much less than interpopulation diversity.

The tendency for strong subdivision in organelle genomes compared with nuclear genes has also been noted in several studies of mitochondrial DNA in animals (e.g., DeSalle *et al.* 1987; Crease, Lynch and Spitze 1990; Hale and Singh 1991) and recently in studies of mitochondrial DNA in pines (STRAUSS, HONG and HIPKINS 1993; DONG and WAGNER 1993). The lower effective population size and lower mutation rate for cpDNA (SLATKIN 1987; BIRKY 1988; BIRKY, FUERST and MARUYAMA 1989), as well as the potential for periodic selection (MARUYAMA and BIRKY 1991), could all importantly contribute to its high subdivision compared with nuclear gene markers. In contrast to mitochondrial DNA in animals and possibly cpDNA in plants with maternal inheritance (BIRKY 1988), however, differences in cpDNA dispersal associated with uniparental inheritance are not likely to be important causes of differential subdivision in pines. Because of predominant paternal inheritance of chloroplasts (WAGNER *et al.* 1991) and monoecy, the rate of migration is not likely to differ greatly between chloroplast and nuclear genomes.

Comparison of allozyme and cpDNA diversity suggests that strong bottleneck events have not caused the high cpDNA subdivision observed. Bishop pine shows almost twice as much population differentiation for allozymes than does either of the other species

(22% *vs.* 12–13%, respectively), yet nearly equal amounts of allelic and gene diversity within populations (MILLAR *et al.* 1988). In the northernmost group of bishop pine—which appears to have migrated furthest (MILLAR *et al.* 1988; HONG 1991)—one population (Trinidad) has substantially reduced genetic diversity (expected heterozygosity of 7%), but the other does not (Mendocino: expected heterozygosity of 12%). Average intrapopulation gene diversity in bishop pine is 11.8%. Yet both populations appear to have a high frequency of the same two cpDNA site mutations. The three species have similar levels of gene diversity within populations, ranging from 12–14% (MILLAR *et al.* 1988). These observations suggest that bottleneck events have not been important—and thus cannot explain—the near fixation of cpDNA variants among our population samples of bishop pine, nor that among species.

Mutation rates differ widely among cpDNA and nuclear genes and may contribute to the greater subdivision observed for cpDNA. BIRKY, FUERST and MARUYAMA (1989) derived an equation for the equilibrium expectation of G_{st} in organelle genomes that included mutation rate: $G_{st} \approx 1/[1 + 2N_{eo}\{L/(L - 1)\}(m_e + \mu)]$, where N_{eo} is the total population size for organelle genes, L is the number of subpopulations, m_e is the migration rate, and μ is the mutation rate. This equation is also suitable for estimating subdivision for nuclear genes if $4N_e$ is substituted for $2N_{eo}$. Assuming migration is effectively zero among species and geographic groups of bishop pine as a consequence of sexual incompatibility and geographic isolation (CRITCHFIELD 1967; MILLAR and CRITCHFIELD 1988), the expression reduces to: $G_{st} \approx 1/[1 + 2N_{eo}\{L/(L - 1)\}\mu]$, indicating that at equilibrium subdivision among isolated populations is a function of population number, size and mutation rate. When G_{st} was evaluated for a variety of population sizes, a range of published cpDNA and allozyme mutation rates, and assuming a large number of populations (*i.e.*, $[L/(L - 1)] = 1$), nearly complete subdivision was found for cpDNA at all population sizes, but intermediate values of G_{st} for allozymes when population sizes were in the vicinity of 150,000 (Figure 6). Although these estimates cannot be regarded as precise as a consequence of probable nonequilibrium conditions, they demonstrate that the combination of a lack of migration, and the low mutation rate and small effective population size for cpDNA, may of themselves be the causes of the high subdivision we observed for cpDNA compared with nuclear gene markers. Heuristically, the low mutation rate for cpDNA contributes to its high amount of subdivision by causing a slow rate of increase of within population diversity once drift differentiates and depletes them of diversity. Low effective population size acts to increase the probability and rate of fixation of new variants due to drift (LI and

FIGURE 6.—Hypothetical estimates of G_{st} for cpDNA and allozymes (nucDNA) at equilibrium with different total population sizes (see text for explanation of model used). Calculations assume a mutation rate in vicinity of 10^{-9} substitutions/site/year for cpDNA (WOLFE, LI and SHARP 1987) and 2×10^{-6} for allozymes (nucDNA) (MUKAI and COCKERHAM 1977); actual mutation rates are shown above plot lines.

GRAUR 1991). Periodic selection could have a similar effect to that of drift by creating population differentiation at the expense of intrapopulation diversity (MARUYAMA and BIRKY 1991).

The distribution and amount of variation for length mutations was very different from that for site mutations. The presence, and degree of polymorphism, of length mutation-prone loci differ widely among species and is likely related to the presence and extent of repetitive DNA (*e.g.*, Blasko *et al.* 1988; Hipkins 1993). As many combinations of repeats could give rise to similarly sized variants, allelic homologies will be difficult to determine—especially on agarose gels. Moreover, genetically identical alleles could arise multiple times via unequal recombination. Depending on the presence, number and size of repeats, the extent of observed polymorphism at a locus could vary dramatically among populations, species, and over time. We therefore contend that variability in mutation rate and problems of interpretation of genotype from phenotype interfere with the ability to reliably estimate population genetic processes—and are likely to be the major reasons for the lack of concordance between our estimates of subdivision from length and site mutations. Length variants derived from highly variable regions should therefore be interpreted cautiously in population genetic and phylogenetic studies of cpDNA.

We observed two trees from different open-pollinated families (HONG 1991) in the San Vicente population of bishop pine that contained an identical major inversion. Although the inversion was not mapped in detail, it appeared identical to a nearly 50-kb inversion observed among two genera of Pinaceae (Figure 2; Pinus and Pseudotsuga: STRAUSS *et al.* 1988). Thus, as suggested previously (STRAUSS and DOERKSEN 1990), it appears that convergent rearrangements can occur in conifers; unless intensively characterized they should be used with caution as phylogenetic

markers. This observation also suggests that the major changes of genome structure observed in "macroevolutionary" studies of conifer genera and families can also be seen during "microevolutionary" surveys; no novel evolutionary events need to be postulated to explain their origin. They exist as infrequent forms in natural populations and will thus occasionally proceed to fixation during or after speciation.

The strong cpDNA differentiation we observed in these pines may be more limited or complex in taxa that are less geographically isolated or are undergoing interspecific hybridization. Bishop pine has developed reproductive barriers among many pairs of populations, as well as distinct morphological and chemical attributes (MIROV *et al.* 1966; MILLAR 1986). In groups that have more extensive gene flow, polymorphisms may be "injected" into otherwise monomorphic, differentiated populations at rates that exceed, or are less than, that for nuclear genes (SCHAAAL, O'KANE and ROGSTAD 1991). Hybridization appears capable of sometimes causing the replacement of one monotypic cpDNA lineage with another, at least when it is maternally inherited (RIESEBERG and SOLTIS 1991). Nonetheless, the strong subdivision we observed in comparison with nuclear genes demonstrates the potential for rapid cpDNA differentiation during reproductive isolation and speciation.

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Note added in proof: Phylogenetic consequences of cpDNA variation are presented in Y.-P. HONG, A. KRUPKIN and S. H. STRAUSS, Chloroplast DNA transgresses species boundaries and evolves at variable rates in the California closed-cone pines (*Pinus radiata*, *P. muricata*, and *P. attenuata*). *Mol. Phylog. Evol.* (in press).

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