

GENETIC VARIATION AT ALLOZYME AND RAPD MARKERS IN *PINUS LONGAEOVA* (PINACEAE) OF THE WHITE MOUNTAINS, CALIFORNIA¹

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We compared genetic diversity estimated from allozymes and from random amplified polymorphic DNA (RAPDs) in a sample of 210 Great Basin bristlecone pines (*Pinus longaeva* Bailey) from three groves in the White Mountains, California, USA. The White Mountains are the most westerly extension of bristlecone pine and home to the oldest known living trees. We assayed two forks of each tree to determine whether they originated from multiple seed caches of the Clark's nutcracker. Despite the limited and fragmented distribution of bristlecone pine, its level of genetic diversity was comparable to that of other pines, but lower than that reported for eastern populations of Great Basin bristlecone pine. Twenty-six of 36 allozymes were polymorphic ($p_{95} = 38.9\%$; $p = 63.0\%$), with observed heterozygosity (H_o) of 0.122 and expected heterozygosity (H_e) of 0.134. The proportion of the total variation among populations (G_{ST}) was only 0.011. The high proportion of trees with multiple stems was not due to germination in seed caches; only six of 210 forked trees had multiple allozyme genotypes. Of the 42 RAPD loci scored, 27 were monomorphic. Genetic diversity for RAPDs was nearly the same as that for allozymes ($p_{95} = 34.1\%$, $H_e = 0.130$). However, the estimates of diversity and differentiation were much higher ($H_e = 0.321$, $G_{ST} = 0.039$) after excluding monomorphic loci.

Key words: bristlecone pine; Clark's nutcracker; genetic diversity; isozymes; Pinaceae; *Pinus longaeva*; RAPDs; seed caches; White Mountains.

Few studies have compared estimates of genetic diversity and genetic structure from the same sample using both allozymes and random amplified polymorphic DNA (RAPD), and more comparisons are needed to draw definitive conclusions regarding the relative merits of these two markers, particularly in highly heterozygous forest trees. Great Basin bristlecone pine (*Pinus longaeva* Bailey) would be an especially interesting species for such a comparison because estimates of genetic diversity in the species are the highest ever reported in the well-studied genus *Pinus* (Hiebert and Hamrick, 1983; Ledig, 1998). Furthermore, genetic diversity of bristlecone pine in the White Mountains, at the western extreme of the species' range (Fig. 1), is an unknown quantity, and these groves contain the oldest known living trees, some in excess of 4500 yr old (Schulman, 1958). In addition to the intrinsic interest of ancient bristlecone pine, it has been suggested that the high frequency of multistemmed trees in the White Mountains, USA, is a result of seed caches by Clark's nutcracker (*Nucifraga columbiana*), and seed caching would be critical to the genetic structure of the species.

Because allozymes and RAPDs each have advantages and disadvantages as markers for genetic variation, the comparison of the two applied to the same set of samples would indicate whether genetic parameters estimated by one were comparable

to those estimated by the other. So far, most comparative studies have used different sets of individuals and/or populations in comparing the utility of allozymes and RAPDs (Karhu et al., 1996; Lee et al., 1997; Aagaard, Krutovskii, and Strauss, 1998; Wu, Krutovskii, and Strauss, 1999). Only a few studies have compared markers using the same samples (Liu and Fournier, 1993; Isabel, Beaulieu, and Bousquet, 1995; Szmidi, Wang, and Lu, 1996; Mitton and Latta, 1997), which is necessary for precise comparison.

Allozyme markers have provided a valuable tool for population genetic studies in natural populations of woody plants (Adams, 1983). They usually exhibit simple Mendelian inheritance and codominant expression, making genetic interpretations easy. In addition, allozyme analysis is relatively fast, inexpensive, and an extensive literature exists (Hamrick, Godt, and Sherman-Broyles, 1992; Ledig, 1998). However, allozymes present limitations, such as highly biased genomic sampling (only genes encoding well-documented, soluble proteins are detectable); a low number of markers, insufficient for examining major portions of the genome; occasional differences between tissues or ontogenetic stages; and difficulty in the standardization of experimental methods from laboratory to laboratory.

The RAPD technique (Williams et al., 1990) has become an increasingly popular tool in genetic studies (Liu and Fournier, 1993; Chong, Yang, and Yeh, 1994; Isabel, Beaulieu, and Bousquet, 1995; Peakall, Smouse, and Huff, 1995; Apostol et al., 1996; Karhu et al., 1996; Szmidi, Wang, and Lu, 1996; Aagaard, Krutovskii, and Strauss, 1998; Wu, Krutovskii, and Strauss, 1999). The RAPD technique can yield a large number of loci, thereby providing a more representative sample of the genome than is possible with allozymes. The use of other DNA-based markers (e.g., RFLPs) is time consuming and technically complex, but RAPDs are the least time consuming and the simplest to apply of the DNA-based markers. Unfor-

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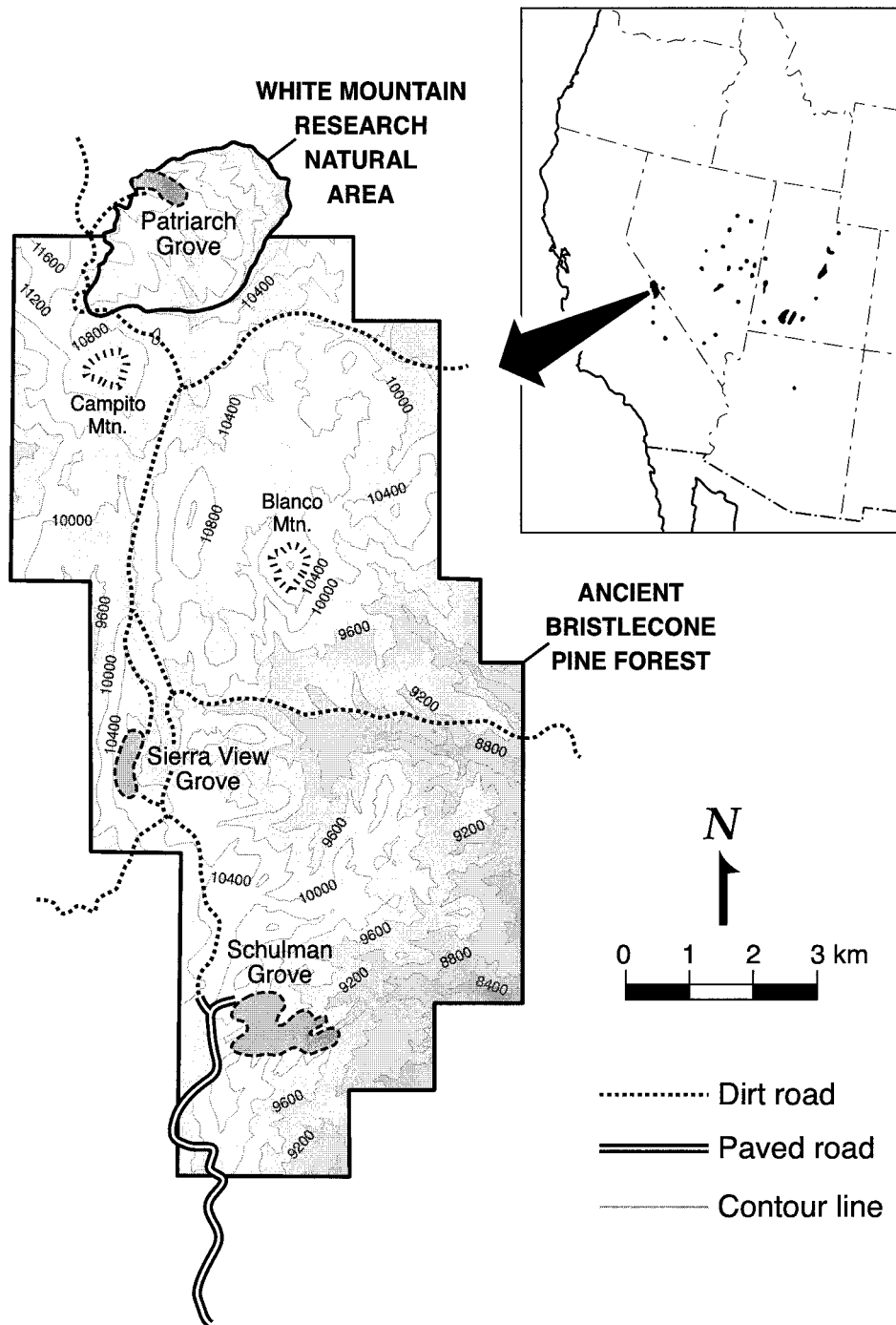


Fig. 1. The natural range of Great Basin bristlecone pine (after Little, 1971) and location of sampled groves in the Ancient Bristlecone Pine Forest, White Mountains, California, USA.

unately, RAPDs also have some significant limitations. One of the most critical limitations for their use in genetic studies is their dominant allelic expression. This characteristic precludes direct estimates of allele frequencies from diploid materials and thus biases the estimates of genetic diversity and genetic differentiation (Lynch and Milligan, 1994; Szmidi, Wang, and Lu, 1996). Furthermore, the reproducibility of RAPDs is lower than that of other genetic markers, and the

literature is relatively limited compared to that of allozyme markers.

Great Basin bristlecone pine has a relatively narrow latitudinal range, but extends from Utah and Nevada westward to eastern California (Lanner, 1999). In California, bristlecone pine grows only in Inyo and Mono counties near the summits of the White and Inyo Mountains, on Sentinel and Telescope peaks in the Panamint Mountains, and as a single tree in the

Last Chance Range. Great Basin bristlecone pines that grow high atop the White Mountains of eastern California exhibit great age, exceeding 4500 yr and even reaching 4700 yr (Cohen, 1998).

Great Basin bristlecone pine is a species believed to have high variability (Hiebert and Hamrick, 1983). Hiebert and Hamrick (1983) studied genetic variation of bristlecone pine in the eastern Great Basin and the western Colorado Plateau (Nevada and Utah), employing allozyme markers. Expected heterozygosity (H_e) was 0.327 and total genetic diversity (H_T) within the range that they sampled was 0.484, well above the average range of values reported for other pines (Ledig, 1998). Based on a larger number of loci, H_e was lower, but still high (Hamrick, Schnabel, and Wells, 1994); in the western Rocky Mountains H_e was 0.237 and in the Great Basin it averaged 0.218.

Life history and ecological characteristics of bristlecones, including large population size, high fecundity, and extreme longevity may explain their high level of diversity (Hamrick and Godt, 1989; Hamrick, Godt, and Sherman-Broyles, 1992). During the last glacial maximum, Great Basin bristlecone pine was apparently widespread at lower elevations throughout the Great Basin, judging from packrat middens. Its present range is restricted to isolated mountaintops. However, within these fragments, it maintains a large population size, its habitat is relatively undisturbed, and it has high fecundity. These factors may have enabled it to maintain a high level of genetic diversity and could play an important role in its evolution.

Judging from the very small seeds, which bear winglike membranes, bristlecone pine seems to rely on wind for seed dispersal, like most of the Pinaceae. However, Lanner (1998, 1999) suggested that Clark's nutcracker was important for seed dispersal of bristlecone pine. He reported that a large proportion of the bristlecone pines in the White Mountains were members of stem clumps that might arise, like those of limber pine (*Pinus flexilis* James) and whitebark pine (*Pinus albicaulis* Engelm.), from nutcracker seed caches (Lanner and Vander Wall, 1980; Schuster and Mitton, 1991; Furnier et al., 1987; Tomback and Linhart, 1990; Rogers, Millar, and Westfall, 1999). In a low-elevation grove in the White Mountains, the Schulman Grove, 46% of the trees were in clumps of two to four, and in a high elevation grove at timberline, the Patriarch Grove, the proportion was 69% (Lanner, Hutchins, and Lanner, 1984).

The objectives of our study were (1) to evaluate levels of genetic diversity in the Great Basin bristlecone pine of the White Mountains, California, with both allozyme and RAPD markers, using the same sets of samples; (2) to examine the consistency of results from allozymes and RAPDs and compare their advantages and disadvantages for population genetic studies in bristlecone pine; (3) to compare genetic variation of bristlecone pines in the White Mountains at the western edge of the species' range to previous reports for the eastern Great Basin; and (4) to determine whether trees with clumped stems were multiple trees arising from Clark's nutcracker seed caches, which could have a major impact on the genetic structure and the mating system of bristlecone pine.

MATERIALS AND METHODS

Plant materials—Three natural groves of Great Basin bristlecone pine (Schulman Grove, Patriarch Grove, and Sierra View Grove; Fig. 1) in the Ancient Bristlecone Pine Botanical Area, White Mountains, California, USA

were selected along a north/south elevational transect in September 1999. The elevation of the Schulman Grove was roughly 3050 m above sea level (a.s.l.), the Sierra View Grove, 3190 m a.s.l., and the Patriarch Grove, 3450 m a.s.l.

From each grove, 70 trees with forked or multiple trunks were selected. The trunks diverged from each other within 1 m of the ground but were fused at the base. The choice of multistemmed trees was to determine whether these stems were actually separate trees that germinated in clumps as a result of seed caching by Clark's nutcracker. Clark's nutcrackers were frequently observed in the groves. Within groves, trees were sampled in close proximity until the goal of 70 trees was reached. Tissue was collected in September 1999 from 190 trees, and the remaining 20 were sampled in September 2000. Buds and foliage were collected from the top of two widely separated trunks, placed in ice chests, and transported to the laboratory within 48 h, where they were stored at 4°C until needed.

Enzyme extraction and allozyme procedures—Tissues were extracted between 1 and 7 d after collection. Bud scales were removed and the buds ground to fine powder with liquid nitrogen and extraction buffer (Cheliak and Pitel, 1984), using a mortar and pestle, and then were stored in microfuge tubes at -80°C. In preliminary trials, enzyme activity showed the best results in the Cheliak and Pitel (1984) extraction buffer with some modifications. When needed, frozen samples were thawed and centrifuged at 1466 rad/s for 2 min. The supernatants were applied to paper wicks for electrophoresis.

Using techniques of starch-gel electrophoresis based on Conkle et al. (1982), the following 21 enzyme systems were assayed: aconitase (ACO), acid phosphatase (ACP), alcohol dehydrogenase (ADH), cinnamyl alcohol dehydrogenase (CADH), fructose 1,6 diphosphatase (FDP), fluorescent esterase (FEST), glutamate dehydrogenase (GDH), glycerate dehydrogenase (GLDH), glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT), glucose 6-phosphate dehydrogenase (G6PD), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), mannose phosphate isomerase (MPI), peroxidase (PER), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), 6 phosphogluconate dehydrogenase (6PGD), shikimate dehydrogenase (SKDH), and UDP glucose pyrophosphorylase (UGPP).

DNA extraction and RAPD procedures—Total genomic DNA was extracted from needles using FastDNA Kit and FastPrep Instrument (BIO 101, Carlsbad, California, USA). Polymerase chain reactions (PCR) were carried out in a volume of 25 μ L with final concentrations of 6 ng of template DNA; 100 μ mol/L of each of the four dNTPs; 2.5 mmol/L $MgCl_2$ (Perkin Elmer, Branchburg, New Jersey, USA); 1 \times PCR reaction buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl; Perkin Elmer); 1 unit of Taq DNA polymerase (Perkin Elmer) and 0.4 μ mol/L of primer. Reactions were overlaid with 15 μ L of mineral oil, and amplifications were performed in a PTC-100 thermocycler (MJR Research, Watertown, Massachusetts, USA) using a period of 1.5 min of initial denaturation at 94°C, followed by 41 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 40°C, 1 min of extension at 72°C, and a final extension step of 10 min at 72°C. Subsequent amplification products were electrophoresed using 1.5% agarose gels with a tris-boric acid-ethylenediamine tetraacetic acid (TBE) buffer at pH 8.0 for 3 h. Gels were visualized by ethidium bromide fluorescence (0.5 μ g/mL) and photographed under UV light. A total of 60 primers (Operon Technologies, Alameda, California, USA) were screened using two representatives from each of the three groves. Fifteen primers that gave clear and reproducible fragment patterns over multiple (at least three) amplifications were selected for final analysis: OPA05 (AGGGGTCTTG), OPA09 (GGGTAACGCC), OPA17 (GACCGCTTGT), OPAF04 (TTGCGGCTGA), OPAF07 (GGAAAGCGTC), OPAF14 (GGTGCGCACT), OPAF16 (TCCCGGTGAG), OPAF20 (CTCCGCACAG), OPAG05 (CCCCTA-GAC), OPAG10 (ACTGCCCGAC), OPAG11 (TTACGGTGGG), OPAG13 (GGCTTGCGGA), OPAG14 (CTCTCGGCGA), OPAG16 (CCTGCGA-CAG), and OPAG19 (AGCCTCGGTT). In order to avoid biasing estimates of polymorphism, the selection of primers for band scoring was dependent only on the clearness and repeatability of RAPD fragments, not on the level of polymorphism.

Genetic inference—For allozyme analysis, the number of loci and alleles were interpreted by drawing on the experience gained in our laboratory from studies of other conifer species (Conkle, 1981) and based on the known subunit structures and cellular compartmentalization of the enzyme (Weeden and Wendel, 1989). Where several zones of activity were observed for a single enzyme, hyphenated numerals following the enzyme abbreviation were used for identification. Thirty-six presumptive loci were scored consistently and used for statistical analyses. Allozymes showed only modest departures from Hardy-Weinberg equilibrium (refer to RESULTS).

The RAPD-PCR fragments were analyzed as alleles by making the following assumptions. First, RAPD products segregate as dominant alleles in a Mendelian fashion. Dominance is widely observed for RAPD fragments in other conifers (Carlson et al., 1991; Lu, Szmidt, and Wang, 1995). Secondly, the groves in this study were in Hardy-Weinberg equilibrium. Third, the RAPD fragments represented the nuclear genome. Finally, fragments of the same apparent size in different trees or groves were homologous.

Estimating genetic parameters—We used the BIOSYS-1 (Swofford and Selander, 1989) computer program to estimate genetic diversity (A , the number of alleles per locus; p , p_{99} , and p_{95} , the proportion of polymorphic loci at the 100%, 99%, and 95% level, respectively; H_o and H_e , the observed and unbiased expected heterozygosities), and Wright's (1965) F statistics (F_{IS} , F_{IT} , and F_{ST}) for allozymes. Effective numbers of alleles were calculated by Hartl and Clark's (1997) method. Deviations of genotype distributions from the Hardy-Weinberg expectations were tested by exact tests (Rousset and Raymond, 1995). The exact P values were estimated by the Markov chain method (Raymond and Rousset, 1995a) using GENEPOP software (Raymond and Rousset, 1995b). Tests for genotypic linkage disequilibrium for 401 two-locus combinations were also performed using the GENEPOP program. Contingency tables for all pairs of loci in each grove were created and then a probability test was carried out for each table using a Markov chain. Nei's (1973) gene diversity (H_T , H_S , and G_{ST}) was obtained using the GST program (Lee and Kim, 1993). The GST program does not correct for small sample size, but that is unimportant in our study because samples were large, composed of 70 trees (140 genomes) in each grove. The degree of genetic isolation among populations was estimated by N_m , the number of migrants per generation. N_m was calculated from Wright's F_{ST} (Wright, 1951), and from the number and frequency of private alleles, the unique alleles found in only one population (Barton and Slatkin, 1986) using the GENEPOP program. We used the computer program BOTTLENECK (Cornuet and Luikart, 1996) to determine whether effective population numbers had been restricted in the recent past. The infinite allele model (Kimura and Crow, 1964) was chosen because empirically it tends to fit allozyme data better than alternatives (Luikart and Cornuet, 1998). The Wilcoxon signed-ranks test was preferred to the sign test because the former has higher power and can be used with as few as four polymorphic loci (Piry, Luikart, and Cornuet, 1999). At least 15 polymorphic loci were available for our samples.

For RAPD markers, the frequency of a recessive allele was estimated by direct count and by Lynch and Milligan's (1994) method using Black's (1997) RAPDBIOS program. The BIOSYS-1 type 3 dataset (Swofford and Selander, 1989) produced by RAPDBIOS (Black, 1997) was used to calculate population genetic parameters such as mean number of alleles per locus (A), percentage of polymorphic loci (p_{95}), and unbiased expected heterozygosity (H_e). In addition, Wright's (1931) F_{ST} , Weir and Cockerham's (1984) θ , and Lynch and Milligan's (1994) F_{ST} were calculated using the RAPDFST program (Black, 1997). For comparison with allozymes, Nei's G_{ST} was also calculated. Finally, N_m was calculated from Wright's F_{ST} .

RESULTS

Allozyme genetic diversity and structure—Ten of 36 loci were monomorphic in all groves sampled (*Cad-1*, *Cad-2*, *Fdp*, *Gld*, *Gpt-1*, *Gpt-2*, *Gpt-3*, *G6pd*, *Mdh-2*, and *Mdh-5*), and 26 loci were polymorphic (Table 1), i.e., a polymorphic locus is one in which any variants were observed in any population. The distribution of allele frequencies was U shaped (Fig. 2),

i.e., most alleles were in high frequency (>0.90) or in low frequency (<0.05), which is the case for most large, natural populations (Chakraborty, Fuerst, and Nei, 1980).

Percentage of polymorphic loci (p_{99}) ranged from 58.3% to 66.7% with a mean of 63.0%, while p_{95} varied between 33.3% and 44.4% with a mean of 38.9%; alleles per polymorphic locus (A) ranged from 2.43 to 2.50 with a mean of 2.45; effective number of alleles (A_e) ranged from 1.21 to 1.25 with a mean of 1.23; observed heterozygosity (H_o) ranged from 0.113 to 0.128 with a mean of 0.122; and expected heterozygosity (H_e) ranged from 0.127 to 0.143 with a mean of 0.134 (Table 2).

In each of the three groves, observed heterozygosity was slightly lower than expected heterozygosity, which suggests a degree of inbreeding. The heterozygote deficiency is also reflected in a population mean of 0.078 for Wright's F_{IS} (Table 3). F_{IS} is a measure of the deviation of the genotypic proportions from Hardy-Weinberg equilibrium at the population level. Positive values suggest a deficiency of heterozygotes relative to the Hardy-Weinberg expectation. Likewise, the value of Wright's F_{IT} was positive (0.088). This also indicates a small heterozygote deficiency at the species level in the White Mountains. These results are in good agreement with the exact test for deviation from Hardy-Weinberg expectations. Sixteen of 63 exact tests for polymorphic loci indicated a significant deviation from the Hardy-Weinberg proportions. In 14 of 16 significant tests, groves had a deficiency of heterozygotes (Table 1).

The tests for linkage disequilibrium showed that only sixteen (3.99%) of 401 two-locus combinations were in disequilibrium, less than would be expected by chance alone. Total genetic diversity (H_T), averaged over 26 polymorphic loci, was 0.186, and H_S , the genetic diversity within groves, was 0.184. G_{ST} and F_{ST} were 0.011, which can be interpreted to mean that 98.9% of the total genetic variation was within populations and only 1.1% was among populations.

Indirect estimates of gene flow between populations were very high. N_m calculated from Wright's F_{ST} (Wright, 1951) and by Barton and Slatkin's method (1986) was 22.5 and 11.5 migrants per generation, respectively.

RAPD genetic diversity and differentiation—Of the 42 RAPD fragments scored, 25 fragments (59.5%) were completely monomorphic. A ranged from 1.3 to 1.4 (Table 2), but because RAPD loci can have only two alleles per locus, A is determined solely by percentage polymorphic loci. The percentage polymorphic loci (p_{95}) ranged from 31.0% to 38.1% with a mean of 34.1%, and the expected heterozygosity (H_e) ranged from 0.122 to 0.146 with a mean of 0.130 (Table 2). When monomorphic loci were excluded from the dataset as recommended by Lynch and Milligan (1994), genetic diversities increased substantially ($A = 1.9 \pm 0.058$, $p_{95} = 84.3\% \pm 8.96$, $H_e = 0.321 \pm 0.033$).

Several different methods of analysis for population differentiation gave congruent trends, although their absolute values sometimes differed (Table 4). Every coefficient of population differentiation (G_{ST} , F_{ST} , and θ) showed that most (96.1 to 98.4%) of the variation resided within groves, as it did in the allozyme analysis. N_m calculated from Wright's F_{ST} was 6.7.

Comparison between allozymes and RAPDs—The estimates of genetic diversity (A , p_{95} , and H_e) for allozymes were nearly the same as those for RAPDs (Table 2). However, when

TABLE 1. Allele frequencies and Weir and Cockerham's (1984) F for 26 polymorphic loci in three groves of Great Basin bristlecone pine. Dashes indicate cases in which the low frequency allele is so rare that expected values and sample size are insufficient for the calculation of F . Probability levels based on Rousset and Raymond's (1995) exact test for deviation from Hardy-Weinberg proportions are * $P \leq 0.05$ and ** $P \leq 0.01$.

Locus	Allele	Grove		
		Schulman	Sierra view	Patriarch
<i>Aco-1</i>	a	0.826	0.833	0.738
	b	0.174	0.167	0.262
	F	0.103	0.176	0.290*
<i>Aco-2</i>	a	0.119	0.125	0.102
	b	0.881	0.875	0.898
	F	0.554**	0.337	0.634**
<i>Acp-1</i>	a	0.933	0.944	0.936
	b	0.067	0.049	0.064
	F	0.000	0.007	0.000
<i>Acp-2</i>	a	0.015	0.007	0.000
	b	0.985	0.993	1.000
	F	-0.008	—	—
<i>Adh</i>	a	1.000	0.957	0.992
	b	0.000	0.043	0.008
	F	—	-0.034	—
<i>Fest</i>	a	0.000	0.000	0.015
	b	0.955	0.950	0.908
	c	0.037	0.025	0.062
	d	0.007	0.025	0.015
	F	-0.033	-0.031	-0.067
<i>Gdh</i>	a	0.695	0.754	0.693
	b	0.305	0.246	0.307
	F	0.306*	0.362*	0.368*
<i>Got-2</i>	a	0.000	0.007	0.000
	b	1.000	0.986	0.964
	c	0.000	0.007	0.036
	F	—	-0.004	-0.030
<i>Got-3</i>	a	0.067	0.056	0.029
	b	0.933	0.944	0.972
	F	-0.065	-0.053	-0.022
<i>Idh</i>	a	1.000	1.000	0.993
	b	0.000	0.000	0.007
	F	—	—	—
<i>Lap-1</i>	a	0.000	0.028	0.057
	b	1.000	0.972	0.943
	F	—	-0.022	0.475*
<i>Lap-2</i>	a	0.963	0.908	0.921
	b	0.007	0.063	0.057
	c	0.030	0.028	0.021
	F	-0.025	0.012	-0.060
<i>Mdh-1</i>	a	0.993	0.965	0.979
	b	0.007	0.035	0.021
	F	—	-0.029	-0.015
<i>Mdh-3</i>	a	0.000	0.000	0.007
	b	0.985	0.923	0.979
	c	0.015	0.077	0.014
	F	-0.008	-0.077	-0.010
<i>Mdh-4</i>	a	0.754	0.824	0.671
	b	0.022	0.035	0.093
	c	0.149	0.099	0.193
	d	0.075	0.042	0.043
	F	0.009	0.048	0.039
<i>Mpi-1</i>	a	0.000	0.000	0.027
	b	1.000	1.000	0.973
	F	—	—	-0.019
<i>Per-2</i>	a	0.343	0.179	0.232
	b	0.597	0.721	0.659
	c	0.060	0.100	0.109
	F	0.321**	0.093	0.224*

TABLE 1. Continued.

Locus	Allele	Grove		
		Schulman	Sierra view	Patriarch
<i>Per-3</i>	a	0.060	0.000	0.014
	b	0.888	0.914	0.768
	c	0.052	0.086	0.217
	F	-0.084	-0.087	-0.273*
<i>Pgi-3</i>	a	0.955	1.000	0.956
	b	0.045	0.000	0.044
	F	-0.039	—	-0.039
<i>Pgm-1</i>	a	0.000	0.014	0.014
	b	0.993	0.979	0.979
	c	0.007	0.007	0.007
<i>Pgm-2</i>	F	—	0.665**	-0.010
	a	0.102	0.070	0.057
	b	0.430	0.482	0.385
	c	0.469	0.447	0.557
<i>6Pgd</i>	F	0.340**	0.290*	0.337*
	a	0.963	0.880	0.957
	b	0.037	0.120	0.043
	F	-0.031	-0.129	0.310
<i>Skd-1</i>	a	0.073	0.080	0.121
	b	0.565	0.464	0.468
	c	0.363	0.457	0.411
<i>Skd-2</i>	F	-0.386**	-0.239	-0.072
	a	0.970	0.979	1.000
	b	0.030	0.021	0.000
<i>Ugp-1</i>	F	-0.023	-0.014	—
	a	0.061	0.038	0.060
	b	0.579	0.621	0.655
	c	0.360	0.341	0.284
<i>Ugp-3</i>	F	0.085	-0.030	-0.055
	a	0.946	0.942	0.949
	b	0.054	0.058	0.051
Mean F	F	0.552**	-0.054	-0.047
		0.083	0.054	0.089

monomorphic loci were excluded from the RAPD data, expected heterozygosity for RAPDs was more than double that for allozymes (0.321 vs. 0.130; Table 2).

Population differentiation (G_{ST}) for RAPDs was more than three times greater than that observed for allozymes (Table 4),

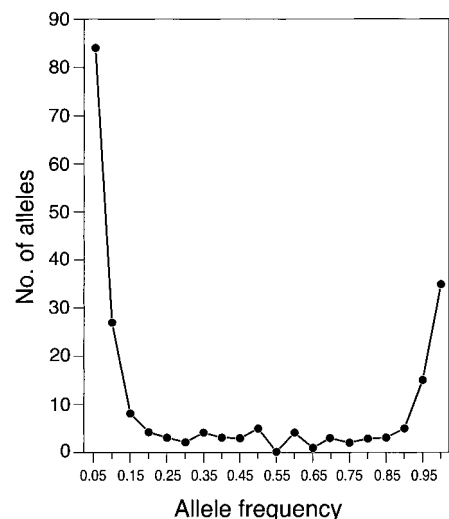


Fig. 2. The U-shaped distribution of allele frequencies in White Mountains bristlecone pine.

TABLE 2. Estimates of genetic diversity in allozyme and random amplified polymorphic DNA markers (RAPDs) of Great Basin bristlecone pine, White Mountains, California, USA, and a comparison with related pines of subsection *Balfourianae* (eastern Great Basin *Pinus longaeva*, *P. aristata*, and *P. balfouriana*) and other conifers, based on all loci and on polymorphic loci alone (in parentheses): number of allozyme loci (*L*), mean sample size per locus (*N*), number of alleles per locus (*A*), effective number of alleles (*A_e*), percentage polymorphic loci (*p*; a locus was considered polymorphic if more than one allele was detected), percentage polymorphic loci at 95% criterion (*p₉₅*), observed heterozygosity (*H_o*), expected heterozygosity (*H_e*) ± the standard error. * indicates indirect RAPD frequencies based on the criterion of Lynch and Milligan (1994).

Species populations	Allozymes										RAPDs		
	<i>L</i>	<i>N</i>	<i>A</i>	<i>A_e</i>	<i>p</i>	<i>p₉₅</i>	<i>H_o</i>	<i>H_e</i>	<i>A</i>	<i>p₉₅</i>	<i>H_e</i>		
<i>Pinus longaeva</i>	36												
Schulman		64.6	1.83 (2.43)	1.22	58.3	33.3	0.113	0.127 ± 0.031	1.4 (1.9)	38.1 (94.1)	0.146 ± 0.031 (0.359)		
Sierra view		62.7	1.94 (2.43)	1.21	63.9	44.4	0.126	0.131 ± 0.029	1.4 (1.9)	33.3 (82.4)	0.122 ± 0.028 (0.302)		
Patriarch		67.2	2.00 (2.50)	1.25	66.7	38.9	0.128	0.143 ± 0.032	1.3 (1.8)	31.0 (76.5)	0.122 ± 0.030 (0.303)		
Mean		65.2	1.92 (2.45)	1.23	63.0	38.9	0.122	0.134	1.4 (1.9)	34.1 (84.3)	0.130 (0.321)		
Species		194.5	2.11 (2.54)	1.23	72.2		0.122	0.135					
<i>Pinus longaeva</i>	14			1.49	78.6			0.327					
<i>Pinus longaeva</i>	27												
Rocky Mt.			2.39		69.2			0.237					
Great Basin			2.42		63.8			0.218					
<i>Pinus aristata</i>	22				46.4			0.139					
<i>Pinus balfouriana</i>	23				57.6			0.208					
<i>Pinus attenuata</i>	36		1.55			40.3		0.11	1.48	48.2	0.15 (0.17*)		
<i>Pinus radiata</i>	32		1.76			47.9		0.14	1.50	49.8	0.17 (0.23*)		
<i>Pinus muricata</i>	32		1.49			40.6		0.11	1.39	38.6	0.13 (0.16*)		
<i>Pinus sylvestris</i>	20												
Svartberget								0.262			0.356 (0.482*)		
Korpilombolo								0.276			0.298 (0.480*)		
<i>Pinus densiflora</i>	23		2.3			65.2		0.261	2.0	94.6	0.392		
<i>Pseudotsuga menziesii</i>	20							0.15			0.19 (0.22)		
<i>Picea mariana</i>	13		2.2			69.2		0.300	1.9	82.1	0.321		

Note: Data as follows: *Pinus longaeva* from the eastern Great Basin (Hiebert and Hamrick, 1983); *P. longaeva* from the western Rocky Mountains in Utah and from montane islands in the Great Basin (Hamrick, Schnabel, and Wells, 1994); *P. aristata* and *P. balfouriana* (unpublished data from Hiebert and Hamrick, 1983); *P. attenuata*, *P. radiata*, and *P. muricata* (Wu, Krutovskii, and Strauss, 1999); *P. sylvestris* (Szmidt, Wang, and Lu, 1996); *P. densiflora* (Lee et al., 1997); *Pseudotsuga menziesii* (Aagaard, Krutovskii, and Strauss, 1998); *Picea mariana* (Isabel, Beaulieu, and Bousquet, 1995).

TABLE 3. F statistics for polymorphic allozymes in bristlecone pines of the White Mountains, California, USA.

Locus	F_{IS}	F_{IT}	F_{ST}
<i>Aco-1</i>	0.084	0.095	0.012
<i>Aco-2</i>	0.488	0.488	0.001
<i>Acp-1</i>	-0.065	-0.064	0.001
<i>Acp-2</i>	-0.013	-0.007	0.005
<i>Adh</i>	-0.039	-0.017	0.021
<i>Fest</i>	-0.056	-0.049	0.007
<i>Gdh</i>	0.338	0.341	0.004
<i>Got-2</i>	-0.029	-0.015	0.014
<i>Got-3</i>	-0.059	-0.053	0.005
<i>Idh</i>	-0.007	-0.002	0.005
<i>Lap-1</i>	0.302	0.315	0.020
<i>Lap-2</i>	-0.029	-0.020	0.009
<i>Mdh-1</i>	-0.028	-0.022	0.006
<i>Mdh-3</i>	-0.061	-0.037	0.023
<i>Mdh-4</i>	0.024	0.039	0.016
<i>Mpi-1</i>	-0.028	-0.009	0.018
<i>Per-2</i>	0.212	0.225	0.016
<i>Per-3</i>	-0.186	-0.140	0.039
<i>Pgi-3</i>	-0.047	-0.031	0.015
<i>Pgm-1</i>	0.271	0.272	0.003
<i>Pgm-2</i>	0.315	0.320	0.007
<i>6Pgd</i>	-0.018	0.005	0.023
<i>Skd-1</i>	-0.234	-0.226	0.007
<i>Skd-2</i>	-0.027	-0.017	0.009
<i>Ugp-1</i>	-0.006	-0.002	0.004
<i>Ugp-2</i>	0.142	0.142	0.000
Mean	0.078	0.088	0.011

but the absolute difference was small (0.039 vs. 0.011). As a consequence, N_m calculated from allozymes was greater than that for RAPDs (22.5 vs. 6.7).

Genetic differences between forks—We found forks that differed in allozyme genotypes at three or more loci in 6 of 210 forked bristlecone pines (Table 5), three from the Schulman Grove and three from the Sierra View Grove. This indicates that the stems arose from different seeds. All the other 204 forked trees were likely the result of branching of a single individual, although in five of these, forks differed at a single allozyme locus. We tentatively interpreted these latter as chimeras for somatic mutations (S. W. Lee, F. T. Ledig, and D. R. Johnson, unpublished manuscript) and not the result of seed caches.

DISCUSSION

Allozymes—Genetic variation in bristlecone pine of the White Mountains was slightly lower ($H_{es} = 0.135$) than that reported previously for pines ($H_{es} = 0.157$ from 93 entries;

TABLE 4. Estimates of genetic differentiation among populations of Great Basin bristlecone pine and other conifers based on allozyme and random amplified polymorphic DNA markers (RAPDs): Nei's (1973) G_{ST} ; Wright's (1931) F_{ST} ; Weir and Cockerham's (1984) θ ; and Lynch and Milligan's (1994) I_{ST} .

Species marker	G_{ST}	F_{ST}	θ	I_{ST}
<i>Pinus longaeva</i>				
Allozymes	0.011	0.011		
RAPDs	0.039	0.036	0.025	0.016
<i>Pinus attenuata</i>				
Allozymes	0.13			
RAPDs	0.24	0.23	0.37	0.37
<i>Pinus radiata</i>				
Allozymes	0.08			
RAPDs	0.18	0.18	0.26	0.26
<i>Pinus muricata</i>				
Allozymes	0.17			
RAPDs	0.29	0.29	0.41	0.42
<i>Pinus sylvestris</i>				
Allozymes	0.022			
RAPDs	0.019			
<i>Pseudotsuga menziesii</i>				
Allozymes	0.05			
RAPDs	0.05			
<i>Picea mariana</i>				
Allozymes		0.033		
RAPDs		0.053		

Note: Data for *Pinus longaeva*, present study; for *P. attenuata*, *P. radiata*, and *P. muricata* from Wu, Krutovskii, and Strauss (1999); for *P. sylvestris* from Szmidt, Wang, and Lu (1996); for *Pseudotsuga menziesii* from Aagaard, Krutovskii, and Strauss (1998); for *Picea mariana* from Isabel, Beaulieu, and Bousquet (1995).

Hamrick, Godt, and Sherman-Broyles, 1992). Genetic diversity at the population level ($H_{ep} = 0.134$) was very similar to the average for other pine species ($H_{ep} = 0.136$; Hamrick, Godt, and Sherman-Broyles, 1992). However, genetic variation of the eastern Great Basin bristlecone pine was substantially greater (Hiebert and Hamrick, 1983; Hamrick, Schnabel, and Wells, 1994). Foxtail pine (*Pinus balfouriana* Grev. et Balf.), another close relative in section *Paracembra*, subsection *Balfouriana*, to which bristlecone pine belongs (Krüssmann, 1985), is found in the high Sierra Nevada, just across the valley from the White Mountains, and its level of genetic variation is similar to that of bristlecone pine from the eastern Great Basin. Foxtail pines, like bristlecone pines, grow high on mountain slopes near timberline, but require more moisture and do not reach as great an age. The closest relative of Great Basin bristlecone pine is the Rocky Mountain bristlecone pine (*Pinus aristata* Engelm.), which grows in less arid environ-

TABLE 5. Great Basin bristlecone pines that showed multiple allozyme genotypes between forks at three or more loci; N is the number of loci at which forks differed in genotype.

Grove	Tree	N	Loci
Schulman			
	4	8	<i>Got-3, Lap-2, Mdh-4, Per-2, Per-3, Pgm-2, Skd-1, Ugp-1</i>
	24	3	<i>Mdh-4, Pgm-2, Ugp-1</i>
	27	3	<i>Per-2, Pgm-2, Skd-1</i>
Sierra View			
	35	7	<i>Mdh-4, Per-2, Per-3, Pgm-2, 6Pgd, Skd-1, Ugp-1</i>
	41	4	<i>Mdh-3, Per-2, Per-3, Skd-1</i>
	57	5	<i>Acp-2, Gdh, Per-2, Skd-1, Ugp-1</i>

ments in the Rocky Mountains of Colorado and New Mexico, and its level of diversity was similar to that of White Mountain bristlecone pine (Table 2).

Species with restricted and discontinuous distributions like Great Basin bristlecone pine often have low genetic diversity relative to more widespread species with similar life histories and ecological characteristics (Hamrick and Godt, 1989; Hamrick, Godt, and Sherman-Broyles, 1992). Nevertheless, Hiebert and Hamrick (1983) reported an extremely high heterozygosity of 0.37 for Great Basin bristlecone pine, although Hamrick, Schnabel, and Wells (1994) estimated a value of 0.223. Either report is high for conifer species. Hiebert and Hamrick (1983) explained high heterozygosity in bristlecone pine by reference to several factors, including (1) high outcrossing rate, (2) large population size, (3) high fecundity, and (4) microhabitat adaptation in a spatially and temporally heterogeneous environment.

The extremely high estimates of genetic variation in Hiebert and Hamrick (1983) may result from scoring only 14 loci, whereas we used 36 in our study. The fewer the loci, the greater the probability of miscalculating H_e if it is intended to characterize average heterozygosity for the entire genome (Mitton and Pierce, 1980). Hamrick, Schnabel, and Wells (1994) used 27 loci and estimated an H_e of 0.219 for bristlecone pine on montane islands of the Great Basin.

It is likely that the genetic diversity of White Mountains bristlecone pine is actually lower than that of eastern Great Basin bristlecone pines. In fact, J. L. Hamrick (University of Georgia, Athens, Georgia, USA, personal communication) found H_e of 0.127 for the Schulman Grove and 0.143 for the Patriarch Grove, based on 27 allozyme loci. These estimates are exactly the same as ours. The bristlecone pines in the White Mountains are the most westerly extension of the species and are peripheral isolates. Their genetic diversity might be lower than that of the central populations due to the influences of random drift in small isolates, due to severe directional selection in ecologically marginal habitats, which may reduce variation (Furnier and Adams, 1986), or due to founder effects during species migration. Several allozyme studies have shown lower genetic diversity in marginal populations (e.g., Guries and Ledig, 1982; Furnier and Adams, 1986) and in founder populations (e.g., Ledig, 2000).

However, there is no evidence of genetic drift or recent bottlenecks in bristlecone pine in the White Mountains. Populations that have experienced a recent reduction of their effective population size generally show a correlated reduction of allele numbers and gene diversity (H_e) at selectively neutral polymorphic loci, but allele number is reduced more rapidly than gene diversity (Cornuet and Luikart, 1996; Luikart and Cornuet, 1998). Thus, in a population recently reduced in size and/or a population that has only recently expanded after a reduction in size (i.e., a bottlenecked population), the Hardy-Weinberg equilibrium heterozygosity (H_e) is higher than the expected heterozygosity in an equilibrium population (H_{eq}), calculated from the observed number of alleles under the assumption of a constant-size (equilibrium) population (Cornuet and Luikart, 1996; Luikart and Cornuet, 1998). In a population at mutation-drift equilibrium (i.e., the effective size of which has remained constant in the recent past), there is approximately an equal probability that a locus shows a gene diversity excess or a gene diversity deficit. The Cornuet and Luikart test detected no excess heterozygosity ($H_e > H_{eq}$) in any of the bristlecone pine groves. The U-shaped distribution of allele frequencies (Fig. 2) and the near absence of linkage disequi-

librium also indicate that random genetic drift has been relatively unimportant in determining the population structure of bristlecone pines in the White Mountains. Under the influence of genetic drift, allele frequencies tend first toward a uniform distribution and then, in the extreme, to fixation and a complete lack of polymorphism (Wright, 1969). In addition, based on the current number of reproductive individuals (cone-bearing trees) in each grove, genetic drift would not seem to have much importance unless the effective population size (N_e) was actually much smaller than the census number. Consequently, we conclude that bristlecone pine in the White Mountains has not recently experienced a bottleneck leading to genetic drift. The reason(s) for the differences between estimates of genetic variation in bristlecone pines from the White Mountains and the eastern Great Basin must reside in the more distant past.

Differentiation among populations in bristlecone pines of the White Mountains, as measured by allozymes ($G_{ST} = F_{ST} = 0.011$), was lower than that in other pines (Hamrick, Godt, and Sherman-Broyles, 1992; Ledig, 1998). All the populations observed in this study were sampled from a single mountain range and separated by a maximum of only ~13 km. This may be sufficient to explain the low level of population differentiation. Nevertheless, Hiebert and Hamrick (1983) also reported a low value (0.038) for G_{ST} in the eastern Great Basin bristlecone pine, and their samples spanned more than 320 km. They hypothesized continuity among bristlecone pine stands during the Pleistocene glacial periods to explain the low degree of population differentiation. However, in a later study, G_{ST} among montane island populations in the Great Basin was estimated as 0.169 (Hamrick, Schnabel, and Wells, 1994).

N_m calculated from Wright's F_{ST} (Wright, 1951) and by Barton and Slatkin's method (1986) was 22.5 and 11.5 migrants per generation, respectively. In either case, rates of gene flow are either now or were in the recent past too high to permit differentiation by random genetic drift.

In all groves of bristlecone pine in the White Mountains, observed heterozygosity was slightly lower than expected heterozygosity. Wright's F_{IS} of 0.078 (Table 3) indicated a deficiency of heterozygotes on average. Similar results were reported for Great Basin bristlecone pine in Utah and Nevada (Hiebert and Hamrick, 1983). A mixed mating system might result in excess homozygosity. If we assume that F_{IS} is the equilibrium inbreeding coefficient (F_e) the outcrossing rate, t , can be estimated by an indirect method (Allard, Jain, and Workman, 1968): $t = (1 - F_e)/(1 + F_e)$. An outcrossing rate of $t = 0.86$ for this study and $t = 0.80$ for Hiebert and Hamrick's (1983) study would result in the observed inbreeding coefficients at equilibrium. The outcrossing rate of Great Basin bristlecone pine has not been measured directly, but rates in the neighborhood of 0.82 to 0.90 have been observed in pinyon pines which, like bristlecone pine, grow as widely spaced trees (Ledig et al., 1999, in press).

The Wahlund effect may be another explanation for excess homozygosity (Wahlund, 1928). In each grove, our sampling scheme was to choose 70 forked trees in close proximity. However, despite the attempt to minimize distance between sampled trees, they were distributed over wide areas, perhaps 350 m between the extremes. Consequently, if family structures are present in bristlecone pine populations of the White Mountains, the deficiency of heterozygotes may be due to the Wahlund effect. High estimates of F for *Aco-1*, *Aco-2*, *Gdh*, *Lap-1*, *Per-2*, *Per-3*, *Pgm-2*, *Skd-1*, and *Ugp-3* in at least some groves may suggest selection at these loci.

A number of pine species with wingless and nearly wingless seeds are dependent on birds for dispersal (Tomback and Linhart, 1990) and commonly occur in clumps of several stems growing from the same point (Furnier et al., 1987; Lanner, 1988). The multilocus allozyme genotypes of clumped white-bark pine stems have shown them to be genetically distinct, indicating that the different stems within a clump are separate individuals that arose from different seeds deposited in bird caches (Furnier et al., 1987). In North America, the principal birds involved in this mutualism are the nutcrackers (*Nucifraga caryocatactes* and *N. columbiana*) and the pinyon jay (*Gymnorhinus cyanocephalus*). Clark's nutcracker is generally considered to prefer large, wingless seeds of pines, but it also feeds on smaller, winged seeds of such other pines as ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) and Rocky Mountain bristlecone pine (Lanner, 1988).

Because bristlecone pine is highly clumped in natural populations and Clark's nutcracker was observed in bristlecone pine stands, Lanner (1988) and Lanner, Hutchins, and Lanner (1984) suggested that bristlecone pine regenerates from seed caches of Clark's nutcracker. We did not find strong evidence from allozyme data to support this hypothesis. Only 6 (2.9%) of 210 multistemmed bristlecone pine showed multilocus genotypic differences (≥ 3 loci) in the White Mountains.

Lanner, Hutchins, and Lanner (1984) observed that bristlecone pines in the White Mountains were more highly forked in the Patriarch Grove (69%) than in the Schulman Grove (46%). From this result, Lanner (1988) concluded that bristlecone pine regenerated far more frequently from Clark's nutcracker seed caches than from wind dispersal on rigorous high-elevation sites. There is no strong physical evidence to support the hypothesis; no one has reported direct observations of bristlecone pine seeds actually being harvested by nutcrackers in the White Mountains, nor has any cone damage of the type caused by nutcrackers been reported. The presence of Clark's nutcrackers in the bristlecone pine stands of the White Mountains may be largely due to the attraction of single-leaf pinyons (*Pinus monophylla* Torr. et Frem.), which occur nearby, and the nearly wingless-seeded limber pines, which occur within the low-elevation bristlecone pine stands, not to the bristlecone pines themselves.

We could find no clumped trees with distinct multiple genotypes in the high-elevation Patriarch Grove but, conversely, we found three trees showing multiple genotype differences between forks in the low-elevation Schulman Grove and three in the mid-elevation Sierra View Grove. Even the "Patriarch," a famous, large tree with several fused stems, 11.2 m in circumference and over 1500 yr in age, had the same multilocus genotype in stems from the opposite sides of the crown. In conclusion, the proportion of tree clumps arising from multiple seeds (i.e., nutcracker seed caches) in the White Mountains may not be as high as Lanner (1988) estimated. The high frequency of stem clumping in the White Mountains bristlecone pine may be due, to a great extent, to trees damaged near the ground by harsh environmental conditions (e.g., strong winds) rather than germination of multiple seeds in a nutcracker's cache. Almost all of the seedlings and young trees we observed in the White Mountains had a single stem rather than multiple stems, indicating that most regeneration is from single seeds.

Comparison of RAPDs and allozymes—The genetic diversities estimated from RAPD data in bristlecone pine are com-

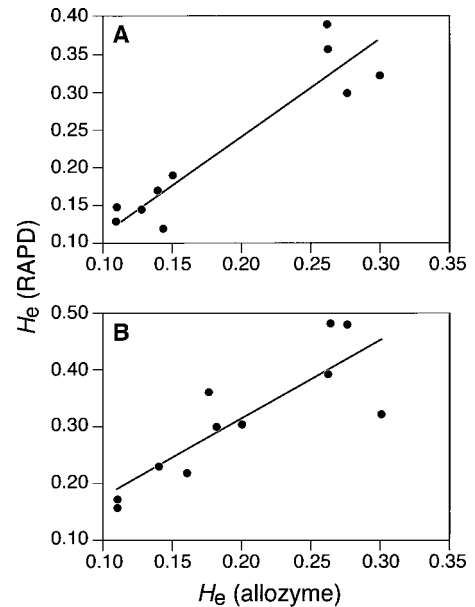


Fig. 3. The correlation of H_e based on allozymes with H_e based on RAPD (random amplified polymorphic DNA) markers for several pine species (data from Table 2) for both monomorphic loci and polymorphic loci (A) and for polymorphic loci only (B).

parable to those of California closed-cone pines (*Pinus attenuata* Lemm., *P. radiata* D. Don, and *P. muricata* D. Don; Wu, Krutovskii, and Strauss, 1999) that have restricted ranges, but lower than those of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco; Aagaard, Krutovskii, and Strauss, 1998), black spruce (*Picea mariana* (Mill.) B. S. P.; Isabel, Beaulieu, and Bousquet, 1995), Japanese red pine (*Pinus densiflora* Siebold et Zucc.; Lee et al., 1997), and Scots pine (*Pinus sylvestris* L.; Szmidi, Wang, and Lu, 1996), most of which are wide-ranging. This tendency is congruent with the results for allozymes (Table 2).

In woody plants, RAPDs generally show similar or higher levels of polymorphism than isozyme markers (Liu and Furnier, 1993; Isabel, Beaulieu, and Bousquet, 1995; Karhu et al., 1996; Szmidi, Wang, and Lu, 1996; Lee et al., 1997; Aagaard, Krutovskii, and Strauss, 1998; Wu, Krutovskii, and Strauss, 1999). The gross patterns of genetic variation for RAPDs and allozyme markers are similar; e.g., Mosseler, Egger, and Hughes (1992) reported low levels of RAPD variation in red pine (*Pinus resinosa* Ait.) from Newfoundland, which is consistent with its monomorphic allozyme profiles. Although few studies have assessed both allozyme and RAPD variation in conifers, Table 2 and Fig. 3 show a positive correlation between genetic diversity estimated from allozymes and RAPDs.

Our estimates of genetic variability based on RAPDs are nearly identical to those based on allozymes (Table 2). However, when monomorphic loci are excluded from both RAPDs and allozymes, following Lynch and Milligan's (1994) recommendation, the expected heterozygosity (H_e) averaged over all populations for RAPDs was much higher than that for allozymes (0.321 vs. 0.134; Table 2). The indices of population differentiation obtained from RAPDs were also higher than those for allozymes (Table 4).

Several factors must be considered when using RAPD markers for estimating population genetic parameters in dip-

oids, especially in outcrossing organisms. Because RAPDs are dominant markers, gene frequency estimates for RAPD loci derived from diploid material are less accurate than those obtained with codominant markers (Lynch and Milligan, 1994; Szmidi, Wang, and Lu, 1996). When only diploid material is available and the null homozygote is not detected, a locus is scored as fixed for the fragment presence even though some of the samples may carry the recessive, null allele. This outcome is even more likely when the population size and the frequency of the null allele are very low. In addition, if there is an excess of heterozygotes relative to Hardy-Weinberg expectations, the frequencies of the null alleles are likely to be further biased downward. This will lead to an underestimate of genetic diversity. The latter, downward bias is unlikely in the case of bristlecone pine since we observed heterozygote deficiency.

Lynch and Milligan (1994) suggested that RAPD analysis be restricted to loci with a null homozygote frequency greater than $3/N$ (N = the number of individuals sampled in a population) in order to avoid biased estimates of genetic parameters. This criterion has been applied in several studies (Szmidi, Wang, and Lu, 1996; Aagaard, Krutovskii, and Strauss, 1998; Wu, Krutovskii, and Strauss, 1999). However, this restriction results in excluding all the monomorphic loci, tends to reject loci that are highly homozygous, and may be satisfied for only a few highly polymorphic loci. Therefore, use of Lynch and Milligan's (1994) correction causes substantial overestimates of genetic diversity measures as shown in the present study as well as in other studies (Isabel, Beaulieu, and Bousquet, 1995; Szmidi, Wang, and Lu, 1996; Wu, Krutovskii, and Strauss, 1999).

The pruning of loci based on Lynch and Milligan's (1994) criteria also affects among-population gene diversities (population differentiation) because the loci that are rejected may differ from population to population. This rejection can exclude many population-specific loci and highly differentiated loci. It can therefore drive the indices for population differentiation downwards (Szmidi, Wang, and Lu, 1996; Wu, Krutovskii, and Strauss, 1999). The pruning of loci implies that more loci (i.e., more primers) and more individual samples (two to ten times more) are needed in RAPD analysis than in a survey involving more conventional codominant markers such as allozymes. Thus, RAPDs would be much more costly and more time-consuming to assay than allozyme markers. Even with large samples and large numbers of primers, estimates of genetic parameters are still likely to be biased in complicated ways, whether or not the pruning procedure is applied. Other methods for handling dominant markers (e.g., Zhivotovsky, 1999) might prove more suitable for analysis of genetic diversity using RAPDs.

Another factor that can inflate estimates of genetic diversity based on RAPDs in diploid tissue is that only the monomorphic loci for dominant alleles (presence of a band) can be observed. Loci monomorphic for recessive alleles (absence of a band) cannot be observed, and this will result in an overestimate of genetic diversity. This may be one possible explanation for higher estimates of variability for RAPDs than for codominant markers in several studies (Isabel, Beaulieu, and Bousquet, 1995; Szmidi, Wang, and Lu, 1996; Aagaard, Krutovskii, and Strauss, 1998; Wu, Krutovskii, and Strauss, 1999). Liu and Furnier (1993) also called attention to this problem.

Regardless of the factors discussed above, RAPDs should be more sensitive markers than allozymes for detecting vari-

ation because of their molecular genetic characteristics: RAPDs result primarily from amplification of noncoding DNA and are subject to a weaker degree of selective constraint than allozymes (for more details, see Aagaard, Krutovskii, and Strauss, 1998 and Wu, Krutovskii, and Strauss, 1999). Computer simulations (Krutovskii et al., 1999) to determine how dominance and biallelism affect the estimation of genetic parameters have supported this hypothesis (Aagaard, Krutovskii, and Strauss, 1998; Wu, Krutovskii, and Strauss, 1999).

Allozymes and RAPDs provided similar estimates of genetic diversity and genetic structure in Great Basin bristlecone pine from the White Mountains, at least when monomorphic loci were included in analysis of RAPDs. RAPDs may be useful in population genetic studies as well as in genetic conservation (Wu, Krutovskii, and Strauss, 1999). In simulations, sample sizes as low as 30 to 50 provided adequate estimates of genetic diversity, depending on the level of variation, number of loci, and population structure (Krutovskii et al., 1999). Samples of ~ 100 are safer. If sample size is small and only diploid tissues are analyzed, RAPD analysis can produce seriously biased estimates of genetic parameters. Codominant allozyme markers still appear most suitable for many types of population genetic studies in plants (Isabel, Beaulieu, and Bousquet, 1995; Peakall, Smouse, and Huff, 1995), and caution should be taken when estimating population genetic parameters using RAPD markers.

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