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A STEEP CLINE IN *PINUS MURICATA*

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Clines, including hybrid zones, have long been studied empirically and theoretically, especially for the opportunity they present to study evolutionary forces (Sumner, 1929; Haldane, 1948; Barber and Jackson, 1957). Recent theoretical studies have emphasized that clines may be important in speciation (summarized in Endler, 1977). This emphasis has motivated mathematical modelling of gene flow and selection (Endler, 1973; Slatkin, 1973; Nagylaki, 1975, 1976), and has renewed interest in the origin and evolution of steep clines (Endler, 1977; Moore, 1977).

Steep clines may evolve due to differential selection along a geographic gradient, hybridization of formerly separate populations, or a combination of these factors. The cause for the clinal origin may be apparent, as in differential selection of metal tolerance in grasses (Antonovics et al., 1971), or in hybridization of hooded and carrion crows in Europe (Mayr, 1963). Occasionally the cause is less apparent, as in *Cepaea* area-effects, where evidence for selection gradients or hybridization is lacking (Jones et al., 1977).

A steep cline occurs in bishop pine (*Pinus muricata* D. Don, subsection *Oocarpae*, Critchfield and Little, 1966), a species with a discontinuous coastal and island distribution in California and Baja California. In northern California, a continuous population of bishop pine extends 180 km from Ft. Ross to Ft. Bragg (Fig. 1). Several morphological and biochemical characteristics change abruptly at Sea Ranch, 27 km north of Ft. Ross. Green foliage, typical of all southern populations, replaces, over approximately 2 km, the blue foliage typical of all northern populations (Duffield, 1951). Distinct differences in the shape and waxiness of the chambers above the stomata determine foliage color. Geographically coincidental with this change is an abrupt transition in the composition of the monoterpene fraction of xylem resin (Mirov et al., 1966). South of the transition, delta-3-carene predominates, whereas to the north, alpha-pinene is the major terpene. Genetic analyses in related species have shown that single or a few loci control stomatal traits and monoterpene composition (Stockwell and Righter, 1946; Forde, 1964). Trees with intermediate terpene composition and stomatal type occur over approximately 2 km. In a major investigation of morphological variation in the species, Duffield (1951) found no other differences between blue and green populations of this region.

Neither ecological nor genetic factors readily explain the origin or maintenance of this steep cline. The coastal environment between Ft. Ross and Ft. Bragg is remarkably uniform in climate, topography, vegetation, and soil pattern. No environmental source for selection is apparent. Critchfield (pers. comm.) found no reproductive barriers in controlled crosses between trees in contiguous blue and green stands, although other populations of this species are isolated by strong reproductive barriers (Critchfield, 1967).

In this study I used variability at allozyme loci in conjunction with ecological information to investigate gene flow and origin of the cline in bishop pine. Using a marker locus I mapped the genotype distribution in standing trees and embryos, and postulate unidirectional pollen flow across the cline. From this and other evidence, interpretations regarding clinal origin and maintenance are proposed.

MATERIALS AND METHODS

Electrophoretic assays were made on haploid female gametophytes and diploid

embryos extracted from freshly germinated seeds. These tissues have been used repeatedly in conifer allozyme research, and the enzyme systems and loci expressed are well known. Bishop pine has predominantly closed (serotinous) cones, so seeds can be collected any time of the year. In all collections, I sampled either current-year or one-year-older cones. To reduce the probability of relatedness between trees, I collected from trees separated by more than 100 m. I scored sample trees and at least ten additional trees in each stand for stomatal type. "Green" (G) stands had all trees with green-type stomata; "blue" (B) stands had all trees with blue-type stomata; and "transition" (T) stands had both types in the same stand.

Immersion in boiling water for 10 sec and drying at approximately 28 C opened the serotinous cones. Seeds were soaked overnight in cold water (5 C) and germinated in petri dishes in an incubator (22 C) until the radicles emerged.

A preliminary study surveyed allozyme variability in samples of green and blue trees considerably south and north of the transition. I collected cones in April 1978 from: (a) 50 trees from five green stands (G1–G5) more than 15 km south of Sea Ranch, and (b) 45 trees from two blue stands (B3, B4) more than 20 km north of Sea Ranch (Table 1). Extracts from one gametophyte per tree were subjected to starch gel electrophoresis (Conkle, 1972). Fourteen enzyme systems were assayed: (a) buffered with tris citrate at pH 8.3—alcohol dehydrogenase (ADH), esterase (β-EST), leucine aminopeptidase (LAP), phosphoglucomutase (PGM), peroxidase (PER), tetrazolium oxidase (TO); (b) buffered with tris citrate at pH 8.8—glutamate-oxaloacetate transaminase (GOT), glucose-6-phosphate dehydrogenase (G6PD), acid phosphatase (ACPH), glutamate dehydrogenase (GDH), catalase (CAT); and (c) buffered with tris citrate at pH 6.2—malate dehydrogenase (MDH), 6-phosphate-glucose dehydrogenase (6-PGD), isocitrate dehydrogenase (IDH). The scored loci segregate according to Mendelian expectations in related conifers

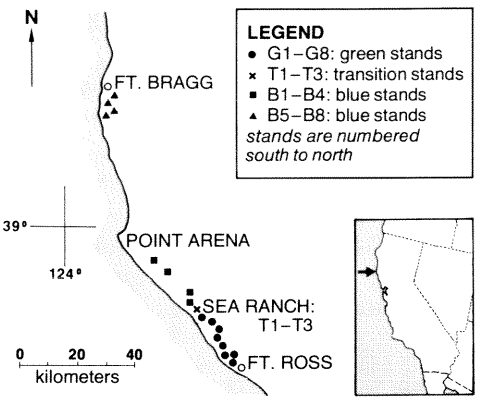

FIG. 1. Location of bishop pine stands sampled in northern California.

(Conkle, 1981). When enzymes had more than one scored locus, I numbered the loci by their relative mobilities, the lowest number (1) designating the locus that migrated the farthest.

A more intensive study sampled trees in the transition area, as well as additional blue trees near Ft. Bragg. Cones were collected in August and September 1978 and July 1979 from 108 trees in green (G6–G8), blue (B1, B2, B5–B8), and transition (T1–T3) stands (Table 1).

I assayed only GOT-1 in the intensive study since preliminary work had shown that it was the most useful marker for blue and green populations. Electrophoretic segregation in gametophytes from 25 heterozygous trees confirmed the Mendelian nature of GOT-1: departure from the expected 1:1 segregation was not significant. GOT-1 was assayed in five gametophytes and two embryos for every tree. Genotype frequencies of standing trees were estimated from five gametophytes per tree. The probability of misclassifying a heterozygote as a homozygote in this case is small (0.06; Morris and Spieth, 1978).

For each tree, I analyzed the genotypes of two embryos with their respective gametophytes. Since the maternal contribution to the embryo is genetically identical to the gametophyte, the paternal contribution can be determined by subtraction.

I recorded onset and duration of flow-

TABLE 1. *Seed collections and enzyme assays performed.*

Stand	Foliage type	Number of trees	Collection date	Tissues[1] assayed	Enzymes[2] assayed
G1	green	5	April 1978	1g	all
				4g, 2e	GOT-1
G2	green	8	April 1978	1g	all
				4g, 2e	GOT-1
G3	green	6	April 1978	1g	all
				4g, 2e	GOT-1
G4	green	15	April 1978	1g	all
				4g, 2e	GOT-1
G5	green	16	April 1978	1g	all
				4g, 2e	GOT-1
G6	green	10	September 1978	5g, 2e	GOT-1
G7	green	7	September 1978	5g, 2e	GOT-1
G8	green	11	September 1978	5g, 2e	GOT-1
T1	mixed	10	July 1979	5g, 2e	GOT-1
T2	mixed	10	September 1978	5g, 2e	GOT-1
T3	mixed	7	September 1978	5g, 2e	GOT-1
B1	blue	9	September 1978	5g, 2e	GOT-1
B2	blue	10	September 1978	5g, 2e	GOT-1
B3	blue	29	April 1978	1g	all
				4g, 2e	GOT-1
B4	blue	16	April 1978	1g	all
				4g, 2e	GOT-1
B5	blue	4	August 1978	5g, 2e	GOT-1
B6	blue	14	August 1978	5g, 2e	GOT-1
B7	blue	4	August 1978	5g, 2e	GOT-1
B8	blue	10	August 1978	5g, 2e	GOT-1

[1] Per tree; g = gametophyte, e = embryo.
[2] See list of enzymes, Table 2.

ering in male and female strobili during 1980 in 22 blue and 24 green trees at the University of California's Russell Reservation, 16 km east of Berkeley. These trees originated as open-pollinated seed collected in 1966 from populations between Ft. Ross and Ft. Bragg. The trees were planted in 1968 as part of a common garden experiment, and many have been sexually mature for several years. Phenological observations in the native stands were made periodically.

The National Climatic Center provided wind-direction data collected at the Pt. Arena Weather Station, 30 km north of the transition.

RESULTS

Of the 14 enzyme systems assayed in gametophytes, 26 loci were scored (Table 2). Eight loci were polymorphic: GOT-1 had one allele common in the blue population, and another allele common in the green population; LAP-1, GOT-2, MDH-2, ADH, and PER-1 had one allele with frequencies greater than 0.75 in both populations, and; LAP-2 and β-EST had two alleles in approximately equal frequencies in both populations. Since allele frequencies in GOT-1 were significantly different between blue and green populations, it was used as a marker.

Multi-locus enzymatic differentiation within and between green and blue populations was measured by Nei's (1972, 1973) diversity and identity statistics. With the exception of GOT-1, green and blue populations are very similar enzymatically (Table 3). When GOT-1 is excluded from calculations, $\bar{G}_{st}$ (0.029) compares with the

TABLE 2. *Allele frequencies of 50 trees south of the cline (green) and 45 trees north of the cline (blue).*

Locus	Allele[1]	Frequencies		Locus	Allele[1]	Frequencies	
		Green	Blue			Green	Blue
Lap-1	*1*	0.16	0.12	*Mdh-3*	*1*	1.00	1.00
	2	0.00	0.12	*Adh*	*1*	0.02	0.00
	3	0.84	0.76		*2*	0.00	0.06
Lap-2	*1*	0.54	0.48		*3*	0.96	0.94
	2	0.46	0.52		*4*	0.02	0.00
Got-1	*1*	0.96	0.24	*Pgm*	*1*	1.00	1.00
	2	0.04	0.76	*Acph-1*	*1*	1.00	1.00
Got-2	*1*	0.96	0.90	*Acph-2*	*1*	1.00	1.00
	2	0.04	0.10	*Per-1*	*1*	0.90	0.98
Got-3	*1*	1.00	1.00		*2*	0.10	0.02
Got-4	*1*	1.00	1.00	*Per-2*	*1*	1.00	1.00
G-6pd-1	*1*	1.00	1.00	*Per-3*	*1*	1.00	1.00
G-6pd-2	*1*	1.00	1.00	*Cat*	*1*	1.00	1.00
Idh	*1*	1.00	1.00	*To-1*	*1*	1.00	1.00
Gdh	*1*	1.00	1.00	*To-2*	*1*	1.00	1.00
6-Pgd-1	*1*	1.00	1.00	*β-Est*	*1*	0.33	0.42
6-Pgd-2	*1*	1.00	1.00		*2*	0.67	0.58
Mdh-1	*1*	1.00	1.00				
Mdh-2	*1*	0.92	1.00				
	2	0.08	0.00				

[1] Alleles at locus are numbered according to migration rates: 1 = fastest, 4 = slowest.

lowest values in comparable conifer studies (Brown and Moran, 1981; Guries and Ledig, 1981; Yeh, 1981).

A steep cline in GOT-1[1] frequencies in standing trees occurs at Sea Ranch. Figure 2 summarizes the allele frequencies of GOT-1[1] in both standing trees and in the pollen contribution to embryos. In standing trees, allele frequencies averaging 0.97 in the green stands decreased to 0.23 in the blue stands over only 2.3 km. The gradient in the pollen allele frequencies was not as steep (Fig. 2): allele frequencies averaged 0.89 in green stands and 0.25 in blue stands.

When analyzed by Chi-square tests, allele frequencies in green stands as a group were homogeneous both among standing trees and among pollen. Blue stands were similarly homogeneous, with the exception of stand B1. In this stand, the pollen frequency of GOT-1[1] was significantly higher than pollen frequency of other blue stands. Transition stands T2 and T3 were homogeneous, whereas T1 had significantly higher GOT-1[1] frequencies in both standing trees and pollen than T2 and T3. Based on these homogeneities, I used average frequencies in subsequent analyses.

Differences in GOT-1[1] frequencies between standing trees and pollen were tested using an arcsine transformation (Sokal and Rohlf, 1969). Frequencies of GOT-1[1] in

TABLE 3. *Measures of gene diversity, genetic identity and genetic distance in bishop pine.*

Statistics[1]	26 loci		25 loci (excl. *Got-1*)	
	Green stands	Blue stands	Green stands	Blue stands
$\bar{H}_c$	0.064	0.075	0.062	0.067
$\bar{D}_{cs}$	0.005	0.006	0.006	0.004
$\bar{H}_t$	0.085		0.069	
$\bar{G}_{st}$	0.141		0.029	
I	0.974		0.998	
D	0.026		0.002	

[1] $\bar{H}_c$ = average gene diversity within stands within blue or green populations.
$\bar{D}_{cs}$ = average interstand gene diversity within blue or green populations.
$\bar{H}_t$ = average total gene diversity in both populations.
$\bar{G}_{st}$ = relative interpopulational gene diversity between composite blue and composite green populations.
I = Nei's genetic identity between composite blue and composite green populations.
D = Nei's genetic distance between composite blue and composite green populations.

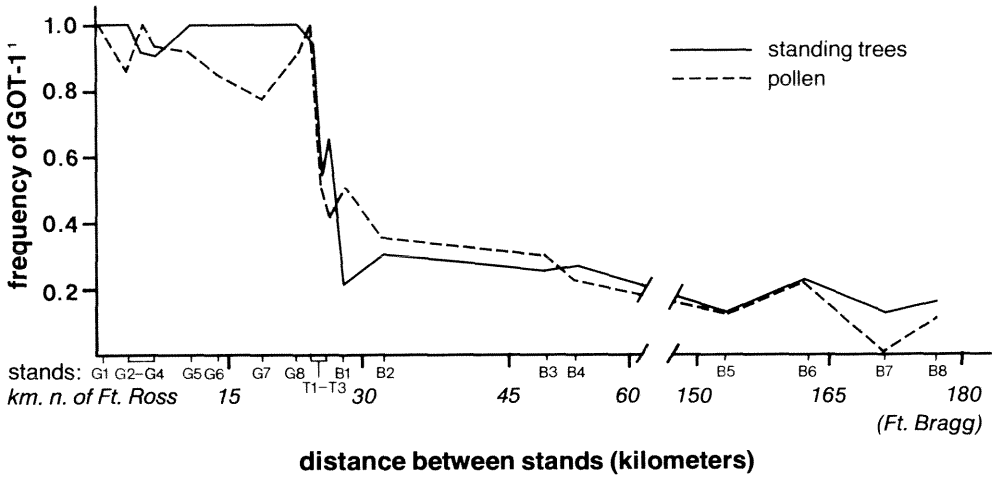

FIG. 2. Cline in frequency of *Got-1*[1] in bishop pine.

green stands (G1–G8) differed significantly in standing trees (0.97) and pollen (0.89). Differences in allele frequencies between standing trees and pollen in transition and blue stands were not significant.

Genotypes of GOT-1 and stomatal type were associated in the transition area. Of 19 green trees, 15 were homozygous for GOT-1[1], four were heterozygous, and none was homozygous for GOT-1[2]. By contrast, of seven blue trees, none was homozygous for GOT-1[1], four were heterozygous, and three were homozygous for GOT-1[1].

Blue trees flowered earlier than green trees in the Russell Reservation plantation (Fig. 3). Modal dates for pollen shedding were 15 days apart (23 April and 8 May). Nevertheless, pollen shedding in the populations overlaps greatly. The periods of female-strobilus receptivity also differed, with modal dates 20 days apart (13 April and 3 May). No overlap between populations occurred. Seven days separated the end of female receptivity in blue trees and its onset in green trees. No green pollen was shed during the period of blue female-strobilus receptivity, but blue pollen was shed almost throughout the entire period of green female-strobilus receptivity. Phenology did not differ significantly in stands within blue or green populations. Observations in natural stands during 1978–1981 were consistent with results in the plantation.

DISCUSSION

The cline in bishop pine is atypical for conifers both because of its steepness and because of the apparent absence of an associated environmental gradient. The steepest intra-specific clines previously reported in conifers extended for tens to hundreds of kilometers (Ledig and Fryer, 1972; Adams, 1975). Inter-specific hybrid zones in conifers may be narrow but the pattern of variation often is not clinal (Wright, 1976). In general, clines in conifers cover large distances, and follow broad environmental and climatic gradients (e.g., Langlet, 1936; Hamrick and Libby, 1972; Conkle, 1973; Griffin and Ching, 1977).

Despite the large differences in stomatal form, terpene composition, and flowering, the blue and green bishop pine populations are little differentiated enzymatically. Only GOT-1 allele frequencies differ significantly in blue and green populations; the remaining loci appear unaffected by the cline.

Gene Flow.—The disparity in GOT-1[1] allele frequencies between standing trees and pollen may be attributed to pollen flow

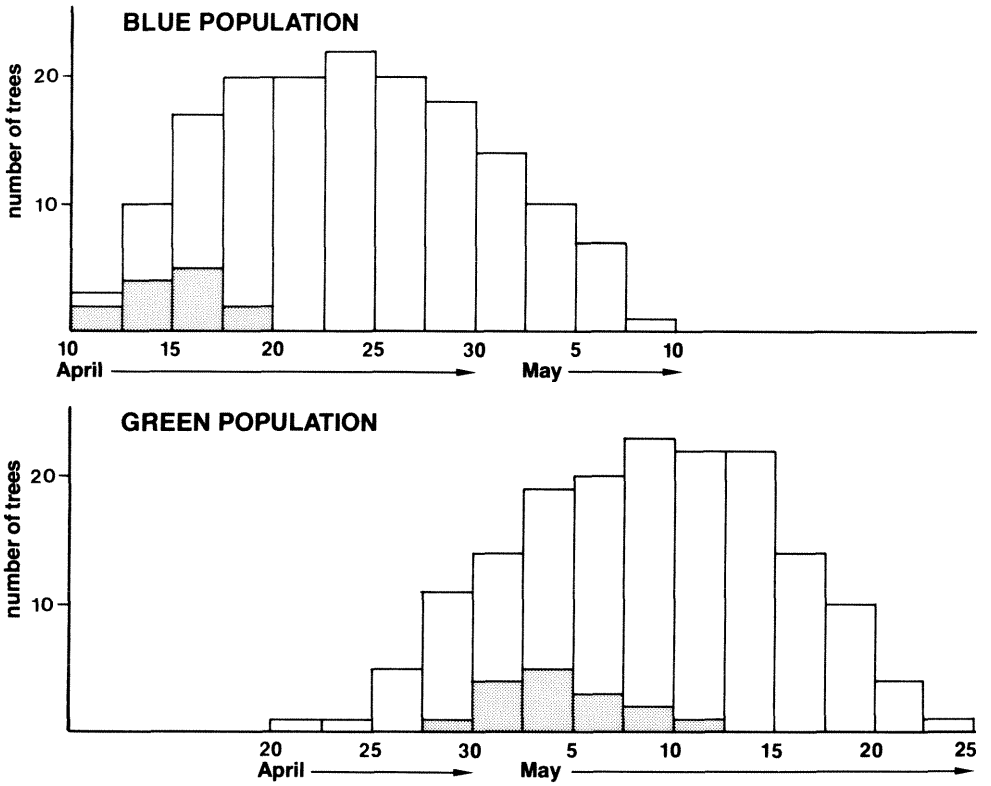

FIG. 3. Distribution of pollen shedding (open bars) and female strobile receptivity (shaded bars) in 22 blue and 24 green trees at Russell Reservation, April and May, 1980.

across the cline. In five of the eight green stands, the allele frequency of pollen and embryos was significantly less than that of standing trees. Pollen flow of GOT-1^2 alleles from northern blue stands to southern green stands would create this disparity. Similarly, Camin and Ehrlich (1958) credited the recurring disparity between juvenile and adult banding frequencies in *Natrix sipedon* to gene flow.

Wind direction and phenology support the north to south gene flow interpretation of the allozyme data. Sixty-five percent of the winds during the pollination season on the coast (April and May) are from the NNW and have velocities averaging 13 mph (National Climatic Center, 1980). These winds occur during fair weather and are probably responsible for most of the pollen dispersal. The differences observed in phenology of pollen shedding and female-strobilus receptivity between blue and green populations similarly would favor north to south pollen dispersal and inhibit pollen movement in the opposite direction (Fig. 3).

GOT-1^1 frequencies in pollen increase with distance from the transition in a pattern consistent with pollen flow (Fig. 2). The higher frequencies in pollen from stands T1 and G8 may result from the location of these stands on points in the shoreline near the transition: northwesterly winds would flow over the ocean before reaching these stands, decreasing the potential for interpopulational pollen flow. Since allele frequencies have been estimated from embryo and adult populations, the following single locus estimator approximates the amount of pollen flow

between the blue population and increasingly distant green stands. Only GOT-1 is useful here since no other loci varied significantly between populations. This model has been used in admixture studies in humans (Workman, 1968; Adams and Ward, 1973).

$$m = (p_e - p_g)/(p_b - p_g)$$

where m is the proportion of blue immigrants, p_e is the GOT-1^1 frequency in embryos, p_g is the GOT-1^1 frequency in green standing trees, and p_b is the GOT-1^1 frequency in blue standing trees (average frequency = 0.23). For stands G8 to G1 respectively, m is 0.06, 0.09, 0.09, 0.06, 0.01, 0, 0.09, 0. These values decrease roughly with distance as expected with long distance pollen flow.

Other explanations currently under study for the disparity in allele frequencies between standing trees and pollen include (1) greater production of pollen by trees with GOT-1^2 alleles than by trees with GOT-1^1 alleles, or (2) competitive advantage of pollen with GOT-1^2 alleles in the nucellus, or of embryos with GOT-1^2 alleles over those with GOT-1^1.

If the disparity in allele frequencies between embryos and adult bishop pine trees is due to pollen flow, then a large amount of long distance gene flow occurs in this species. In other conifers, estimates of gene flow are highly variable. Pollen dispersal studies have estimated gene flow as short (less than one km: Colwell, 1951; Wright, 1953; Strand, 1957; Wang et al., 1969; Sarvas, 1967) or long (tens of kilometers: summarized in Lanner, 1966; Koski, 1970). These studies measured pollen transport, whereas actual gene flow may be considerably different. Values calculated in this study estimate effective pollen flow, and are consistent with reports of long distance pollen dispersal in conifers.

Implications for the Origin and Maintenance of the Cline.—Endler (1977) stressed the difficulty in discerning the mode of origin of steep clines once they are established. Several lines of evidence suggest that the cline in bishop pine did not develop by differential selection in continuous populations but evolved following secondary contact of formerly allopatric populations. Theoretically, the lack of evidence for an environmental gradient coupled with the possibility of widespread gene flow argue against non-allopatric development of the cline. Furthermore, paleobotanic, geologic, and climatic evidence indicates a history of repeated fragmentation of bishop pine populations. Abundant fossil cones of bishop pine from central and southern California document frequent shifts in distribution during the late Tertiary and Pleistocene (Axelrod, 1980). Orogenic activity caused a constant change in land-sea relations during the late Tertiary and Pleistocene (Bailey and Jahns, 1954; Bailey, 1966), and Mason (1934) proposed that pine populations were isolated at one or more times on islands created by such tectonic events. Expansions and contractions of pine populations appear correlated with cyclic climatic changes during the Pleistocene (Mason, 1934; Johnson, 1977), and Axelrod (1967*a*, 1967*b*, 1980) attributed a recent fragmentation of pine populations to the drought and heat of the Xerotherm (8000–4000 years before present). Since the Xerotherm, the California climate has become cooler and wetter, and populations of several species are expanding in areas where they are not inhibited by other reasons (Axelrod, 1981). Thus the Tertiary and Pleistocene history of bishop pine is replete with population fragmentation and expansion events, the most recent probable contraction occurring during the Xerotherm.

Thus we may be witnessing in bishop pine a recent meeting of blue and green populations. The fate of the cline is unpredictable from current knowledge, although selection for clinal maintenance is implicated from several clues. First, if pollen flow is long distance (e.g., more than 3 km), then differences in neutral traits between the populations would have become more gradual as the populations met and interbred, and a steep cline (less than 3 km) would never have formed. Second, the disparity in allele frequency between

embryos and mature trees suggests post-embryonic selection, and the association of allozyme genotypes and foliage type in mature trees in the transition suggests selection against hybrid recombinants. The disparity in flowering time of blue and green populations may indicate an early stage in the reduction of gene exchange between the populations. The flowering time of the green population in this study is completely out of an ordered latitudinal sequence that has been observed for the remaining California and Baja California populations (Wilcox et al., 1979; pers. obser. Russell Reservation 1978–1981). The green population of this study flowers last of all the bishop pine populations.

Summary

Two northern California bishop pine populations differing in stomatal form, monoterpene composition, flowering time, and allozyme frequency are separated by a cline of less than 3 km width. Allelic frequencies at a marker allozyme locus in standing trees changed from an average of 0.97 north of the cline to 0.23 south of the cline. Differences in allelic frequencies between mature trees and embryos are attributed to long distance pollen flow across the cline. Data on wind direction and flowering phenology support this hypothesis. Genetic, ecological, and paleontological evidence suggests that the cline resulted from recent contact of formerly isolated populations.

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