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Genetic diversity, structure, and demographic change in tanoak, *Lithocarpus densiflorus* (Fagaceae), the most susceptible species to sudden oak death in California[1]

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Knowledge of population genetic structure of tanoak (*Lithocarpus densiflorus*) is of interest to pathologists seeking natural variation in resistance to sudden oak death disease, to resource managers who need indications of conservation priorities in this species now threatened by the introduced pathogen (*Phytophthora ramorum*), and to biologists with interests in demographic processes that have shaped plant populations. We investigated population genetic structure using nuclear and chloroplast DNA (cpDNA) and inferred the effects of past population demographic processes and contemporary gene flow. Our cpDNA results revealed a strong pattern of differentiation of four regional groups (coastal California, southern Oregon, Klamath mountains, and Sierra Nevada). The chloroplast haplotype phylogeny suggests relatively deep divergence of Sierra Nevada and Klamath populations from those of coastal California and southern Oregon. A widespread coastal California haplotype may have resulted from multiple refugial sites during the Last Glacial Maximum or from rapid recolonization from few refugia. Analysis of nuclear microsatellites suggests two major groups: (1) central coastal California and (2) Sierra Nevada/Klamath/southern Oregon and an area of admixture in north coastal California. The low level of nuclear differentiation is likely to be due to pollen gene flow among populations during postglacial range expansion.

Key words: California forest; conservation; Fagaceae; *Lithocarpus densiflorus*; microsatellites; phylogeography; *Phytophthora ramorum*; sudden oak death (SOD); tanoak.

Disease epidemics caused by exotic forest pathogens can have devastating effects on native ecosystems (Burdon et al., 2006; Desprez-Loustau et al., 2007). The consequences of severe mortality may be far-reaching for ecosystem health if the host species is an important biological component of the habitat and if environmental hazards of fire and erosion are significant risks. This is the case of tanoak [*Lithocarpus densiflorus* (Hook. & Arn.) Rehder, Fagaceae], recently attributed to a new genus [*Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S. Oh] that occupies several forest types in coastal California, southern Oregon, and the Sierra Nevada, Klamath, and Cascade mountains (Fig. 1). In interior northern California, it commonly occurs in shrub form [*Lithocarpus densiflorus* subsp. echinoides (R. Br. Campst.) Abrams]. Tanoak is the most susceptible host to *Phytophthora ramorum* (Werres deCock and Man in't Veld), an exotic pathogen that causes sudden oak death (SOD) (Davidson et al., 2003; Rizzo and Garbelotto, 2003; Rizzo et al., 2005). Although tanoak has not coevolved with *P. ramorum* and is therefore unlikely to have specific resistance genes (Deprez-Loustau et al., 2007; Suarez and Tsutsui, 2008), some degree of resistance, or tolerance may be present as is the case for other exotic pathogen interactions, such as white pine blister rust (*Cronartium ribicola*) (Kinloch et al., 1970) and jarrah dieback (*Phytophthora cinnamomi*) (Stukely and Crane, 1994). Dodd et al. (2005, 2008) have reported genetic-based variability in response of coast live oak (*Quercus agrifolia* Née) to inoculation with *P. ramorum*, and preliminary studies indicate within- and between-population variation in response to inoculation in tanoak (K. Hayden, A. Nettel, R. S. Dodd, and M. Garbelotto, University of California, Berkeley, unpublished data).

The likelihood for evolution of resistance by the host depends strongly on the amount of genetic variation on which selection can act (Crow, 2002), and the level of genetic structuring among host populations should play an important role in the evolutionary trajectory of the host response (Parker and Gilbert, 2004). Therefore, an understanding of genetic structure of susceptible host species is crucial in implementing resistance screening. Where populations are at risk for extinction, as is the case for tanoak, discovery of divergent population lineages will also provide a rationale for conservation priorities. Recent advances in DNA analysis permit studies about past demographic changes in natural populations. Phylogeographic patterns observed in maternal lineages, such as chloroplast DNA in angiosperms, help to infer paleogeographic population processes such as pre-Pleistocene vicariance and late Quaternary population fluctuations caused by past climate change. Different Pleistocene refugial patterns have been detected, from mass extinctions of high latitude populations and restriction to southern refugia (Petit et al., 1993; Brunsfeld et al., 2001; Hewitt, 2004) to in situ refugia for alpine species (Stehlik et al., 2002; Schönswetter et al., 2005), Mediterranean pine (Afzal-Rafii and Dodd, 2007) and sky island taxa in the southwestern United States (DeChaine and Martin, 2005). Surprisingly little is known about plant restrictions during the Quaternary in the California Floristic Province or of subsequent effects of Holocene climate changes on plant distributions and fluctuations in population size. Comparative analysis of animal and plant taxa suggests

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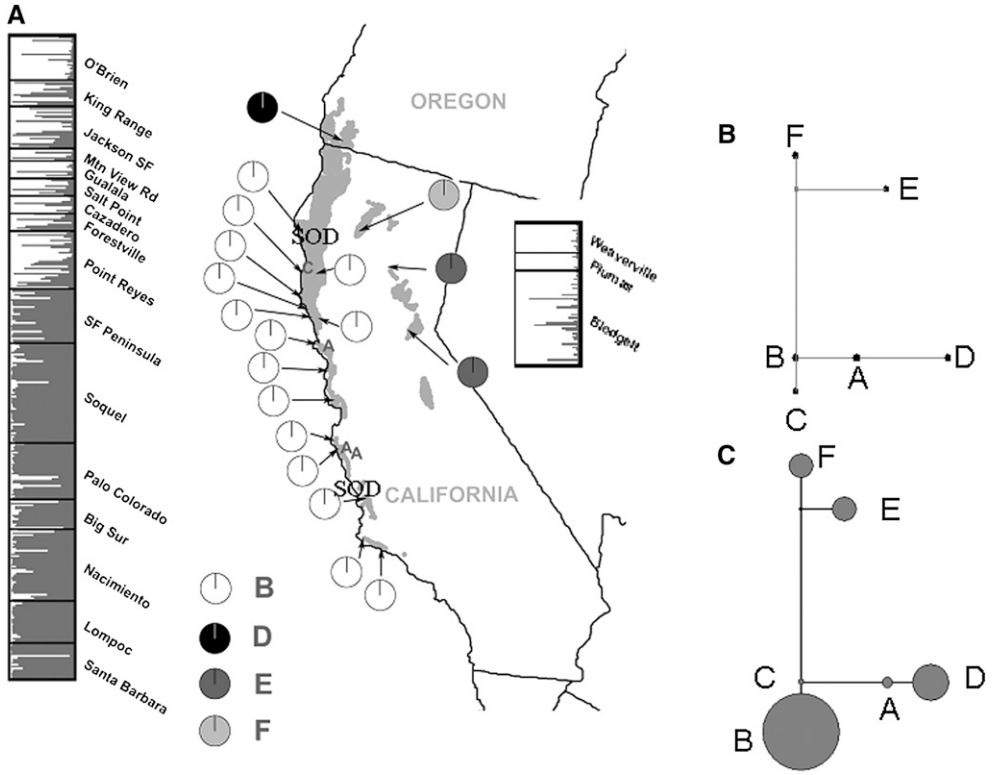

Fig. 1. (A) Distribution of tanoak and sampling locations shown by pie charts denoting composition of chloroplast DNA haplotypes in each population. Rare haplotypes are labeled on the map as A and C. Assignment of individuals to one of two groups based on nuclear microsatellites, as detected using STRUCTURE (Pritchard et al., 2000), is shown by population and drawn as line segments with DISTRUCT (Rosenberg, 2004). (B) Maximum parsimony phylogenetic networks are shown in (B) for chloroplast microsatellite and sequencing of the *trnH* region and, in (C) for chloroplast microsatellites alone, with size of circles at nodes proportional to numbers of individuals. Networks were drawn using NETWORK 3.510 (Polzin and Daneschmand, 2003). Approximate northern and southern limits of confirmed sudden oak death in natural woodlands indicated by SOD. Background map of California in (B) is from Tappeiner et al. (1990).

weaker vicariant structure associated with mountain building in California Floristic Province plants (Calsbeek et al., 2003) and diversification over the last 1 Myr.

The combination of plastid and nuclear DNA studies can provide a powerful complementary approach to detecting ancient to more recent population demographic changes. Chloroplast DNA in most angiosperms is inherited maternally and therefore depends upon seed dispersal. For heavy-seeded species such as tanoak, seed dispersal is likely to be quite limited, and so chloroplast DNA provides a good indication of ancient fragmentation of the species' range. On the other hand, highly polymorphic, codominant markers such as nuclear microsatellites allow for inferences on fluctuations in population size, including founder events and bottlenecks. Because it is impossible to obtain pre- and postevent samples in many natural systems, methods have been developed for estimating the likelihood of past demographic changes from present-day samples. These tests can be broadly grouped into equilibrium-based and coalescence-based approaches. The former depends on detecting departure in the sampled population from expectations of heterozygosity or distributions of allele frequencies in an idealized population in mutation–drift equilibrium (Cornuet and Luikart, 1996; Luikart et al., 1998; Reich and Goldstein, 1998; Garza and Williamson, 2001). Coalescence-based approaches to detect past changes in population size developed by Griffiths and Tavaré (1994) and Kuhner et al. (1998) and further developed by Beaumont (1999) estimate the likelihood of obtaining the sample configuration and are considered more efficient because they capture all the information present in the sampled data.

In this study, we investigated the demographic history of tanoak and how it has influenced genetic diversity and divergence among contemporary populations. The aim is to understand how evolutionary forces have shaped contemporary genetic structure in this species. We anticipate that this knowledge will provide a rationale for strategies for screening for disease resistance and for the identification of management units for conservation and ultimate restoration. The distribution of SOD is continually updated by the California Oak Mortality Task Force, which provides maps of reported and confirmed infection (http://www.cnr.berkeley.edu/comtf/). In March 2009, confirmed occurrences of SOD extended from Plasket Creek in Monterey County, California, north along the coast to Redway in Humboldt County, California. Tanoak is common throughout most of this range. In our study of genetic diversity in tanoak we asked three questions: (1) How is diversity of the maternally inherited chloroplast genome and of the biparentally inherited nuclear genome partitioned throughout the range of tanoak? (2) What inferences can be drawn on the depth of divergent processes in this species? (3) Did populations of tanoak undergo severe reductions in size that could have resulted in loss of genetic diversity?

MATERIALS AND METHODS

Sampling and DNA analysis—Leaves from mature individuals of *Lithocarpus densiflorus* subsp. *densiflorus* were obtained from coastal populations from southern Oregon to Santa Barbara, California, and from the southern Klamath Mountains and the Sierra Nevada (Table 1, Fig. 1). We sampled a total of 447 trees from 19 populations, with an average of 23 trees per population. Leaves were maintained frozen at –20°C. We extracted DNA using a simplified CTAB method (Cullings, 1992).

Chloroplast DNA—Four sources were used to test for polymorphic cpDNA microsatellite loci in tanoak: (1) 10 universal angiosperm primers developed by Weising and Gardner (1991), (2) 14 *Quercus* (Fagaceae) cpDNA microsatellite primers developed by Deguilloux et al. (2003), (3) 14 Fagaceae cpDNA microsatellite primers developed by Sebastiani et al. (2004), and (4) primers directly developed from tanoak cpDNA sequences of the intergenic regions *trnH-trnK*, *trnK1-trnK2*, and *trnC-rpoB*. Loci were first tested for amplification and polymorphism in a panel of 8–12 individuals from different Californian populations by PCR-amplification and electrophoresis on a 6% polyacrylamide gel at 300 V for 4–5 h. Gels stained with ethidium bromide were observed under a UV light transilluminator, and polymorphism was assessed by direct observation of the resulting bands. PCR conditions of the cpDNA intergenic regions and cpDNA microsatellite loci, as well as sequencing procedures are described elsewhere (Nettel and Dodd, 2007). Loci that amplified successfully and appeared to be polymorphic were then amplified for a larger set of samples (24) using one fluorescent-labeled primer and were electrophoresed on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, California, USA) to confirm their usefulness. After testing more than 40 putative loci, we found only five polymorphic cpDNA mononucleotide microsatellites in tanoak (Table 2): *ccmp4* (*atpF* intron, Weising and Gardner, 1999); µ*cd2_p14* (*trnC-ycf6*, Deguilloux et al., 2003); *Cmcs5* (*ndhG-ndhI*, Sebastiani et al., 2004); *KI* (*trnK*), and *BII* (*rpoB*) (new loci developed in this study). The two new mononucleotide repeat loci are $(A)_{11}N_{18}(T)_7$ for *trnK* and $(T)_{10}$ for *rpoB*. The primers developed to amplify them are trnKF2 (5′TTATCTAGCTGTGATTCATCAG) and trnK(HK) from Demesure et al. (1995) for *KI*; and *rpo*BF3 (5′GCACTTAACCTTTTCGCGGATTC) and *rpo*BR2 (5′TCTTTCGCGCTTCACTCG) for *BII*. Sequences of these regions were deposited in GenBank; accessions FJ213617, FJ213616. These five loci were amplified for a total of 291 individuals from the 19 populations sampled; because of haplotype monomorphism in populations, we amplified fewer individuals than were used in nuclear microsatellite analyses.

Because of the homoplasious nature of mononucleotide repeat chloroplast microsatellites (Kelchner, 2000), we further PCR amplified and sequenced 824 bp from the *trnH-His* to the *psbA* genes using the primers HK (Demesure et al., 1995) on one sample for each haplotype and two samples for the most common haplotype B (GenBank accessions FJ213610-FJ213615, FJ213617). This was done to provide additional cpDNA data to confirm patterns observed in the repeat regions. Standard PCR reactions containing bovine serum albumin as adjuvant were performed in a Techne (Cambridge, UK) thermocycler. The Big Dye 3.1 terminator kit (Applied Biosystems) was used for cycle sequencing with the PCR primers. Reactions were precipitated and resuspended in 15 µL of Hi-Di formamide before loading them into the automated sequencer.

Nuclear DNA—Eleven nuclear microsatellite loci developed for tanoak (*LD1*, *LD3*, *LD5*, *LD7*, *LD8*, *LD10*, *LD12*, *LD13*, *LD14*, *LD17*, *LD19*; Morris and Dodd, 2006) were PCR amplified using a fluorescent-labeled primer. Primers were multiplexed in five different groups: (1) *LD1*, *LD3*, *LD7*, *LD10*, *LD14*; (2) *LD5*, *LD17*; (3) *LD12*, *LD13*; (4) *LD19*; and (5) *LD8*. The PCR cocktail contained 1× PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 250 nM of each reverse primer, 250 nM of each fluorescently labeled (FAM or HEX) forward primer, 1 unit of AmpliTaq Polymerase (Invitrogen, Carlsbad, California, US) and approximately 5 ng template DNA in a 20 µL reaction. Forward and reverse primers for locus *LD3* were run at a final concentration of 400 nM, exclusively. Touchdown PCR cycling conditions for all loci were as follows: one cycle at 95°C for 10 min followed by 20 cycles of 45 s at 94°C, 45 s at 58°C (lowering 0.5°C each cycle), and 45 s at 72°C. The final amplification step consisted of 20 cycles of 45 s at 94°C, 45 s at 48°C, and 45 s at 72°C and was followed by a final extension step at 72°C for 45 min. All reactions were performed on a Techne Flexigene thermocycler. We mixed 0.75 µL of PCR product with 8 µL of formamide and 0.5 µL of 500 LIZ size standard (Applied Biosystems), and we electrophoresed this cocktail on an ABI 3730 automated sequencer. We used the programs GENESCAN 3.7 and GENOTYPER 3.7 (Applied Biosystems) to analyze ABI results. We sequenced loci *LD3* and *LD5* because they showed odd-sized series in fragment analyses. The sequences showed that indels within the flanking regions were responsible for the different size series, and so the fragment sizes were adjusted appropriately.

Data analysis—We checked data quality for scoring errors in nuclear and cpDNA microsatellites using the program MICRO-CHECKER (Oosterhout et al., 2004). We used the same program to estimate the probability of the presence of null alleles in nuclear loci. Loci *LD13* and *LD19* had significant values of homozygote excess consistent with null alleles in 16 and 12 populations, respectively. These two loci were excluded from further analysis.

Chloroplast microsatellite alleles were combined into haplotypes, where each amplified locus was considered an independent, but linked, character inherited as a unity. Evolutionary relationships among the haplotypes were explored using the median joining (MJ) network algorithm (Bandelt et al., 1999) employed by the program Network 3.510 (available as freeware from http://www.fluxus-engineering.com/sharenet.htm). The MJ networks were postprocessed with a maximum parsimony (MP) algorithm (Polzin and Daneschmand,

TABLE 1. Characterization of the 19 tanoak populations sampled in California and Oregon. H_e = expected heterozygosity; R_t = allelic richness; N = sample size of nuclear and chloroplast microsatellite survey. Standard errors in parentheses.

				Nuclear microsatellites				Chloroplast DNA	
Population	Code	County	Latitude/Longitude	N_{nuc}	H_e	R_t	Private alleles	N_{chlor}	Haplotype
Lompoc	LOM	Sta. Barbara	34.58/120.50	24	0.45(0.02)	2.39(0.11)	—	20	B
Santa Barbara	SBA	Sta. Barbara	34.50/119.82	20	0.53(0.03)	2.97(0.14)	4	15	B
Pfeiffer	PFE	Monterey	36.25121.78	17	0.50(0.02)	2.89(0.13)	1	8	B
Palo Colorado	PCO	Monterey	36.39/121.90	32	0.55(0.02)	3.18(0.16)	1	20	B(17), A(3)
Nacimiento	NAC	Monterey	35.81120.75	41	0.46(0.03)	2.93(0.14)	1	4	B
Soquel	SOQ	Santa Cruz	37.05121.85	57	0.51(0.01)	2.8(0.13)	—	26	B
SF Peninsula	PEN	San Mateo	37.40122.28	31	0.49(0.02)	2.87(0.11)	3	22	B(21), A(1)
Point Reyes	PRY	Marin	38.06122.80	33	0.5(0.02)	3.07(0.14)	1	19	B
Forestville	FOR	Sonoma	38.47122.90	10	0.59(0.01)	2.87(0.08)	—	10	B
Cazadero	CAZ	Sonoma	38.49/123.06	10	0.54(0.02)	3.23(0.15)	—	10	B, C(1)
Salt Point	SPO	Sonoma	38.56123.32	10	0.53(0.02)	2.87(0.11)	1	10	B
Gualala	GUA	Mendocino	38.76123.52	10	0.55(0.02)	3.09(0.16)	1	10	B
Mtn. View Rd.	FBR	Mendocino	39.43123.80	7	0.56(0.02)	3.17(0.10)	—	7	B
Jackson Forest	JSF	Mendocino	39.35123.61	24	0.55(0.01)	2.94(0.10)	5	33	B
King Range	KRG	Humboldt	40.16124.08	15	0.54(0.02)	3.03(0.14)	1	7	B
O'Brien	OBR	Josephine, OR	42.01123.73	25	0.58(0.02)	3.26(0.14)	3	16	D
Weaverville	WEA	Trinity	40.69122.93	17	0.59(0.03)	3.75(0.19)	4	17	F
Blodgett	BLO	El Dorado	38.73120.75	54	0.58(0.02)	3.37(0.12)	3	23	E
Plumas	PLU	Butte	39.93121.58	10	0.56(0.02)	3.21(0.11)	—	14	E

TABLE 2. Chloroplast DNA haplotypes found in the 19 populations of tanoak sampled. Each letter corresponds to a different haplotype. The last four characters were obtained from the *trnH-His-psbA* region sequence; the other five were collected from microsatellite loci. Binary coding of character 3 corresponds to the presence (1) or absence (0) of a 5-bp indel. GenBank accessions are for the *trnH-His-psbA* sequence.

	Microsatellite loci					*trnH* sequence				
Haplotype	*cmcs5*	*BII*	*KI*	*ccmp4*	*P14*	Substitutions			Indel	GenBank
A	150	118	131	115	152	C	C	G	1	FJ213613, FJ213615
B	150	120	131	115	152	C	C	G	1	FJ213614
C	150	120	131	115	153	C	C	G	1	FJ213614
D	151	118	131	115	152	C	T	T	1	FJ213610
E	151	120	133	117	152	T	C	T	0	FJ213612
F	152	120	132	117	152	C	C	T	1	FJ213611

2003) to remove unnecessary linkages and median vectors. MP networks were constructed for chloroplast microsatellites alone and for combined microsatellite and sequence data. For chloroplast microsatelite networks, the full sample data set was used, providing haplotype frequencies in the output. In constructing MJ networks from the combined microsatellite and sequence data, we downweighted the microsatellite loci to 5 relative to 10 for the sequence characters as suggested by Bandelt et al. (1999).

We obtained the following estimates of genetic diversity and population differentiation at the chloroplast level using CPSSR (Pons and Petit, 1995, 1996): within-population diversity ($_{cp}H_S$), overall genetic diversity ($_{cp}H_T$), global among-population differentiation by allele identity ($_{cp}G_{ST}$), and global among-population differentiation taking into account the allele size ($_{cp}R_{ST}$). We also used CPSSR to test if the observed $_{cp}R_{ST}$ differed significantly from $_{cp}G_{ST}$. This test is used to detect phylogeographic patterns that would be the case if mutation has contributed significantly to population differentiation.

To ascertain the reliability of the estimations based on nuclear microsatellites, we tested deviations from Hardy–Weinberg (HW) equilibrium within each population by the inbreeding fixation index, F_{IS}, with the software FSTAT version 2.9.3.2 (Goudet, 2002). HW equilibrium could not be rejected for any of the populations. We estimated allelic richness with the rarefaction method (R_t) and expected heterozygosity (H_e) with FSTAT to analyze the level of within population genetic diversity.

We used the program STRUCTURE version 2.0 (Pritchard et al., 2000) to infer population structure from the nuclear microsatellite diversity. The admixture model with correlated frequencies was used without prior population information. Preliminary runs revealed a complex hierarchical structure. We inferred population division (number of K populations) by performing 20 independent runs of each K ($K = 1$ to $K = 12$) with a burn-in of 100 000 iterations, and 1 million iterations of the Gibbs sampler. Log-likelihood of the data was recorded for each run and the ad hoc statistic ΔK was calculated following Evanno et al. (2005). Output from STRUCTURE was postprocessed for publication using the program DISTRUCT (Rosenberg, 2004).

To confirm results from STRUCTURE, we performed analyses of molecular variance (AMOVA, Excoffier et al., 1992) using the program ARLEQUIN version 3.11 (Excoffier et al., 2005). Populations were assigned to two, three, or four subgroups following results from chloroplast DNA divergence. Isolation by distance was tested within groups forming the optimal partition using Mantel tests for correlations between matrices of genetic distance (F_{ST}, Weir and Cockerham, 1984) and geographic (Euclidean) distances as implemented in the Isolation by Distance Web Service (IBDWS) version 2.00 (Jensen et al., 2005). Significance of the test statistic Z was assessed by 10 000 random permutations.

We investigated whether mutations at the nuclear microsatellite loci were a significant cause of population differentiation in tanoak using the R_{ST} permutation procedure described by Hardy et al. (2003) as implemented in the program SPAGeDi version 1.2 (Hardy and Vekemans, 2002). Because permutated values of R_{ST} are based on allele identity and ignore allele size, the resulting expected value is equal to F_{ST}. If mutation at the microsatellite loci has contributed significantly to differentiation, the observed value of R_{ST} should be higher than the permuted R_{ST}. Populations that have been isolated for a significant period of time would have accumulated mutations that contribute to their differentiation. Conversely, if populations were isolated for a relatively short period of time (in comparison to the mutation rate) or if gene flow has been important, R_{ST} would not be significantly different from F_{ST} (Hardy et al., 2003).

Past demographic change—We performed equilibrium-based and coalescent-based methods to test for past demographic changes based on nuclear microsatellites. Among equilibrium-based tests, we used the program BOTTLENECK 5.1 (Piry et al., 1999) to identify recent population bottlenecks by comparing observed heterozygosity with the expected heterozygosity in equilibrium populations. This test, developed by Cornuet and Luikart (1996), is based on the notion that, in a bottleneck event, rare alleles are lost faster than common alleles. As rare alleles contribute little to expected heterozygosity, the expectation is that bottlenecked populations would show an increased level of heterozygosity for a given number of alleles relative to equilibrium populations. We tested for a signal of a bottleneck in all sampled tanoak populations under the two-phase model (TPM) with the default 70% single-step mutations and 30% multiple-step mutations. The TPM is intermediate between the infinite allele model and the stepwise mutation model and is likely closer to the true mutation model of most microsatellites (Piry et al., 1999; Williamson-Natesan, 2005). Statistical significance was tested with the Wilcoxon sign-rank test. We also tested combined populations following the STRUCTURE groupings.

We also used two t tests that rely on allele size distribution as described in Reich and Goldstein (1998) to detect overall expansion for the species. A signal of population expansion would be consistent with range expansion after restriction to refugia. The within-locus k-test (Reich and Goldstein, 1998) is based on the assumption that a constant-sized population would present a multimodal distribution of allele sizes, and an expanding population would present a unimodal, more peaked distribution of allele sizes. The differences in the distributions stem from ancient bifurcations of gene lineages (Reich et al., 1999). For this test, the proportion of loci having positive values of the statistic k is estimated, and a binomial distribution with the probability of $k > 0$ set to 0.515 is used to assess significance. The interlocus g-test relies on the broader variance of allele-length distributions at a constant-sized population when compared to an expanding population. Significance of g at the 0.05 level is obtained by comparing the observed value to tabulated values given in Reich et al. (1999). Reich and Goldstein (1998) point out that a signal of expansion would be detected for up to $20N_1$ generations (where N_1 is the ancestral, pre-expansion N_e).

Evidence of past demographic change for the species (all populations combined) was also studied using the coalescence-based Markov chain Monte Carlo approach of Beaumont (1999) as implemented in the program MSVAR version 0.1.4. The software assesses the probability distributions for the demographic and genealogical parameters r, t_f, and θ. The parameter r is the rate of population change defined as the ratio of the current over the ancestral population size ($r = N_0 / N_1$); the parameter t_f is the time in generations since the population started changing in size scaled by the present size of the population ($t_f = t_a/N_0$), and the population parameter θ is defined as twice the present population size times the mutation rate ($2N_0m$). The model uses a strict stepwise mutation model and assumes that the parameters r and t_f are identical among loci, but that θ can vary. We defined broad, uninformative rectangular priors for all the parameters on a $\log_{10}$ scale with the following bounds: $\log_{10}(r)$ [−3, 1], corresponding to an r from 0.001 to 10; $\log_{10}(t_f)$ [−4, 4], corresponding to a t_f from 0.0001 to 10 000; $\log_{10}(\theta)$ [−4, 1], corresponding to θ from 0.0001 to 10, to minimize their effects on posterior distributions. The linear model of population change was adopted because it is recommended to study ancient population changes. We performed eight independent runs with different starting parameters for r (0.01, 0.1) and t_f (0.5, 2.0, 5.0 10.0) with 20 000 thinned updates and a thinning interval of 10 000 steps (total of 2×10^8 update steps). Mixing of the chains was evaluated visually by plotting parameters against time on each run. The first 10% updates of each run were discarded as burnin. The mode and the 90% highest posterior density (HPD) limits of the posterior distributions of the parameters are reported as estimated using the Locfit package (Loader, 2006) implemented in the freely available R software (R Development Core Team, 2005).

RESULTS

Chloroplast diversity—We detected only two (*BII, ccmp4,* μ*cd2_p14*) or three alleles (*Cmcs5, KI*) per locus at chloroplast microsatellite loci, resulting in a total of six haplotypes designated A–F (Table 2). Sequencing of the *trnH-His* to the *psbA* fragment detected four polymorphic sites: one substitution

unique to haplotype D, another substitution unique to haplotype E, a 5-bp insertion unique to haplotype E, and a substitution that separated haplotypes A, B, and C from D, E, and F. Because of the high frequency and wide distributional range of haplotype B (Fig. 1), we sequenced one individual from a central California population and one from a northern California population. Sequences from these two individuals were identical. All but three populations (Palo Colorado, SF Peninsula, and Cazadero) were monomorphic for haplotypes that were regionally well defined. The three polymorphic populations included two rare haplotypes: haplotype A detected in a single individual from the San Francisco peninsula and in three individuals from Palo Colorado and haplotype C detected in a single individual from Cazadero (Fig. 1, Table 1). The cpSSR haplotype network showed the close relationship among coastal California haplotypes A, B, and C and a possible link to the southern Oregon haplotype D (Fig. 1). Haplotypes E (Sierra Nevada) and F (Klamath) were separated by a median vector and five mutations from haplotype B. This phylogenetic relationship did not change when sequence and microsatellite data were combined, even though the microsatellites were downweighted.

Estimates of cpSSR diversity were low, both at the intrapopulation ($_{cp}H_S$ = 0.03) and overall population ($_{cp}H_T$ = 0.40) level. Genetic differentiation based on allele identity was $_{cp}G_{ST}$ = 0.93 and based on allele size was $_{cp}R_{ST}$ = 0.97. The observed level of diversity and differentiation indicate that few unrelated haplotypes dominate the landscape and are fixed or almost fixed in populations. $_{cp}R_{ST}$ was significantly different from $_{cp}G_{ST}$, confirming the importance of distantly related haplotypes in tanoak's cpDNA genetic structure.

Nuclear genetic diversity and structure—Average values of population genetic diversity estimates were 0.54 and 3.05 for observed heterozygosity (H_e) and allelic richness (R_t), respectively. H_e ranged from 0.45 in Lompoc to 0.59 in Weaverville and Forestville. R_t ranged from 2.39 in Lompoc to 3.75 in Weaverville (Table 1). The analysis of molecular variance at the global level was significant with 90.8% of the variance explained by intrapopulation diversity. Differentiation at the global level was relatively low, F_{ST} = 0.09 (99% CI = 0.07–0.11), but significant. Private alleles were detected in 13 of the 19 populations sampled. The highest number of private alleles was found in Jackson State Forest (5), followed by Weaverville (4) and San Marcos (4).

The observed value of R_{ST} (0.05) was not significantly different from the expected value of F_{ST} (P = 0.50) according to the distribution of permuted R_{ST}; therefore, we infer that mutation is much less important than drift and/or gene flow for the observed differentiation in tanoak at the nuclear level.

The ad hoc statistic ΔK, which summarizes our results from 20 independent runs per K (from K = 1 to K =12) in STRUCTURE, detected that tanoak populations can be divided in two major groups (Figs. 1, 2). Assignment of individuals to these two groups shows differentiation between a central coastal California group (from Santa Barbara county to San Francisco peninsula) and a mostly interior group that included almost all samples from the Sierra Nevada, the Klamath Mountains, and southern Oregon. Remaining populations from northern coastal California show an intermixture between these two groups (Fig. 1). AMOVA results provided the highest among group variance component for the two-region partition in support of the STRUCTURE analysis, although Φ_{ST} values differed little among the three regional groupings tested here (Table 3). IBDWS returned

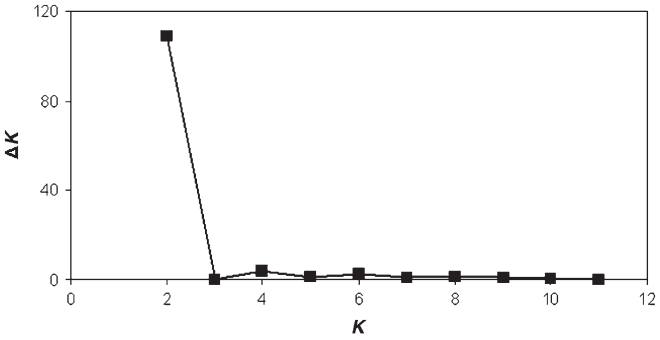

Fig. 2. Determination of the optimum number of groups from STRUCTURE (Pritchard et al., 2000) analysis for nuclear microsatellite loci in tanoak populations, using the plot of ΔK against K, as suggested by Evanno et al. (2005).

a positive Mantel correlation (r = 0.64, $P \leq 0.001$) between genetic distance and geographic distance for California coastal populations representing group 1 from the STRUCTURE output and including admixed coastal populations north of Point Reyes. However, isolation by distance was nonsignificant (r = 0.34, $P \leq 0.074$) for group 2 from the STRUCTURE output that included Sierra Nevada/Klamath, southern Oregon, and admixed north coastal populations (Fig. 3).

Past demographic change—BOTTLENECK detected significant values of heterozygote excess, consistent with a recent bottleneck with the Cornuet and Luikart (1996) test, in three of the 19 tanoak populations sampled: Lompoc ($P < 0.05$), San Marcos ($P < 0.05$), and Forestville ($P < 0.01$). Forestville, however had a low sample size, N = 10. When populations were combined following STRUCTURE groupings, BOTTLENECK failed to detect heterozygote excess or deficiency in any of the groups.

No signal of population expansion was detected using the k and g tests. For the within-locus k test, positive k values were found for only three loci corresponding to a nonsignificant P-value of 0.45. For the interlocus g test, the value of g was 0.39, which was nonsignificant following the table in Reich et al. (1999) for nine loci. Results from the coalescence-based MSVAR procedure supported a long-term trend of decline in tanoak (Table 4). Eight independent runs with different starting parameters resulted in a mode posterior $\log_{10}(r)$ of −1.71 (SE = 0.004) with 90% highest posterior density (HPD) limits from −2.11 (SE = 0.016) to −1.36 (SE = 0.003). These values of $\log_{10}(r)$ correspond to an $r = N_0/N_1$ between 0.008 and 0.044, which is consistent with a long-term reduction of population size with a current population size of 1% and 5% compared to the ancestral population size. The mode of the posterior distribution of $\log_{10}(t_f)$ was 0.02 (SE = 0.006) with 90% HPD limits from −0.15 (SE = 0.003) to 0.23 (SE = 0.004).

DISCUSSION

Disease epidemics in forest ecosystems can have devastating consequences when the susceptible host is a keystone species. A cascade of biotic and abiotic effects can lead to transformation of the ecosystem, imposing permanent ecological, social, and economic costs. Screening for resistance and protection of populations of conservation interest are two priorities in the

TABLE 3. AMOVA of nuclear microsatellite data from tanoak with a partition of populations into (A) two regional groups according to results from STRUCTURE (Pritchard et al., 2000), into (B) three regional groups by treating coastal California populations as two groups (central coastal–south of San Francisco peninsula and north coastal–Point Reyes to the King Range), and (C) four regional groups according to results from chloroplast haplotype diversity.

Source	Sums of squares	Variance components	% Variance components	F_{st} (P_{rand} less than estimate)
A) Two-regions ([Sierran, Klamath and Oregon]; [coastal California])				
Among regions	44.3	0.068	2.1	0.104 (<0.0001)
Among populations (regions)	256.3	0.273	8.3	
Within populations	2567.9	2.935	89.6	
B) Three-regions ([Sierran, Klamath and Oregon]; [north coastal California]; [central coastal California])				
Among regions	61.3	0.059	1.8	0.102 (<0.0001)
Among populations (regions)	239.3	0.274	8.4	
Within populations	2567.9	2.935	89.8	
C) Four-region ([Sierran]; [Klamath]; [Oregon]; [coastal California])				
Among regions	74.5	0.048	1.5	0.100 (<0.0001)
Among populations (regions)	226.0	0.278	8.5	
Within populations	2567.9	2.935	90.0	

early stages of the disease outbreaks. In wild ecosystems, the structure of genetic diversity in the threatened host is rarely known and must be one of the first avenues to be addressed for resource management. Screening for resistance is expensive and time-consuming and can be helped by knowledge of host genetic structure that might indicate the optimal sampling strategy. Conservation is aimed at maintaining existing evolutionary potential through identification of population lineages that may have unique adaptive potential (Moritz, 1994; Crandall et al., 2000). Population lineages are the result of ancient processes that have led to distributional discontinuities and demographic fluctuations including bottlenecks and founder events. Vicariant events can lead to isolation of populations and consequent divergent evolutionary trajectories. Understanding what events have given rise to contemporary population genetic structure is therefore important in designing resistance studies and developing a rationale for conservation strategies. The recent SOD epidemic has indications of becoming a major transforming influence in the coastal forests and woodlands of California and Oregon (Barrett et al., 2006; Meentemeyer et al., 2004), and currently, tanoak is the host that has suffered greatest mortality. Screening of tanoak for resistance has begun (K. Hayden, A. Nettel, R. S. Dodd, M. Garbelotto, University of California, Berkeley, unpublished data), but the rationale for selecting populations for screening is hampered by lack of knowledge of genetic structure of this host species. In this paper, we provide the first report on genetic diversity and structure among populations of tanoak, taking an evolutionary approach to understand divergence among populations. We have detected complementary information from the chloroplast and nuclear genomes that show signals of past demographic change. Differences in overall gene flow and genetic dispersal by seed contribute to the distinct patterns displayed by the two genomes and likely reveal evolutionary forces affecting each genome during different periods in the past. We discuss the evolutionary processes that could have shaped the observed results in tanoak and their implications for conservation efforts in this potentially endangered species.

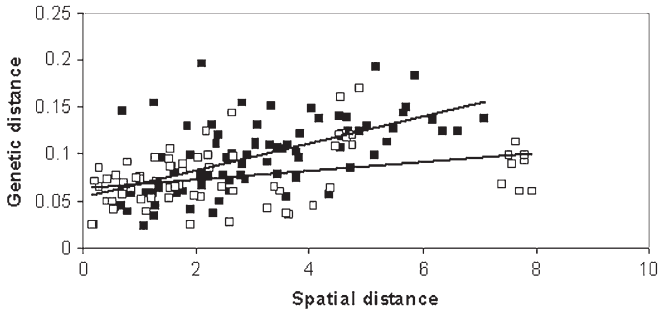

Fig. 3. Relationship between pairwise Weir and Cockerham's (1987) F_{ST} and pairwise Euclidean geographic distances between sampled populations of tanoak. Open rectangles Sierra Nevada/Klamath/southern Oregon and northern coastal California populations (solid regression line), closed rectangles coastal California (dashed regression line) following STRUCTURE (Pritchard et al., 2000) partition.

Genetic diversity and structure—The northern region of tanoak's range (from Humboldt County, California to southern Oregon, Klamath Mountains, and northern Sierra Nevada) presented the highest level of genetic diversity. Here, four cpDNA haplotypes were detected compared with a single haplotype that extended throughout coastal California from Santa Barbara County to Humboldt County. The northern region is characterized by an orographically diverse landscape and an array of habitats within coastal and inland mountain ranges. The discontinuous, but extensive, distribution of tanoak through this region and the effect of past climatic fluctuations could have enhanced isolation and genetic drift leading to the enrichment of regional genetic diversity. We failed to detect any mixture of these four haplotypes in the sampled populations. Since the chloroplast genome is maternally inherited in most angiosperms, and our data on progeny arrays indicate this is also the case for tanoak (R. Dodd, unpublished data), the lack of polymorphic populations suggests that the distance of seed dispersal is limited and that populations have been geographically restricted in the past. The Klamath region of northern California and Oregon has been regarded as a refugial area for many western North American forest trees during the last glacial maximum (LGM) (Whittaker, 1961; Smith and Sawyer, 1988; Soltis et al., 1997). However our haplotype phylogeny suggests a relatively deep divergence between the Klamath and Sierran haplotypes and the remaining haplotypes. This divergence could indicate differentiation of Klamath and Sierran lineages during earlier expansion–contraction phases of the Pleistocene. Finer sampling in the northern range and in the Sierras should shed light on the number of refugia and depth of divergence in the Klamath area and in the secondary contact zone between northern/Sierran lineages and the coastal California lineage. Our chloroplast DNA markers need to be supported by sequence data sampling more of the chloroplast genome. Interestingly, the coastal haplotype B extended over approximately 800 km with only two rare haplotypes occurring interspersed in this range. Either barriers to seed dispersal are less important over this range compared with the northern range, or the haplotype

TABLE 4. Results summary of eight independent runs of MSVAR using nine nuclear microsatellite markers of tanoak. Estimators r (the ratio of the current over the ancestral population size N_0/N_1) and t_f (the number of generations since change in population size scaled by present effective population size t_a/N_0). For details, see text.

	$\text{Log}_{10}(r)$			$\text{Log}_{10}(t_f)$		
Initial values	0.05%	Mode	0.95%	0.05%	Mode	0.95%
$r = 0.01$, $t_f = 0.5$	−2.03	−1.69	−1.36	−0.10	0.070	0.24
$r = 0.01$, $t_f = 2.0$	−2.10	−1.70	−1.36	−0.16	0.039	0.23
r = 0.01, $t_f = 5.0$	−2.01	−1.69	−1.38	−0.14	0.020	0.20
$r = 0.1$, $t_f = 0.5$	−2.10	−1.70	−1.36	−0.15	0.036	0.23
$r = 0.1$, $t_f = 2.0$	−2.07	−1.71	−1.39	−0.17	0.024	0.23
$r = 0.1$, $t_f = 5.0$	−2.04	−1.71	−1.38	−0.14	0.032	0.20
$r = 0.01$, $t_f = 10.0$	−2.42	−1.78	−1.35	−0.17	0.000	0.22
$r = 0.1$, $t_f = 10.0$	−2.11	−1.70	−1.32	−0.13	−0.082	0.31
Mean (SE)	−2.11 (0,016)	−1.71 (0,004)	−1.36 (0,003)	−0.15 (0,003)	0.017 (0,006)	0.23 (0,004)

has existed along the coast for a long time, perhaps in localized pockets. The low level of diversity and high differentiation in cpDNA markers, provide evidence in favor of long-term isolation of lineages within the Sierran and Klamath range of tanoak.

At the nuclear genome, among population differentiation was low ($F_{ST} = 0.09$; $\Phi_{ST} = 0.10$), but within the range commonly recorded for rangewide studies of long-lived tree species (Hamrick et al., 1992). The STRUCTURE and AMOVA analyses indicated an optimal partition into just two groups. These included a southern coastal group south of the San Francisco Bay and an interior/northern (Sierran/Klamath/southern Oregon) group. The intervening coastal populations from Point Reyes to the King Range displayed a mixed population structure. The lack of phylogeographic structure in nuclear DNA (as demonstrated by the R_{ST} vs. F_{ST} test, the isolation by distance (IBD) signal, and the detection of an intermixture zone in the northern coast) indicates lack of strong barriers to gene flow among tanoak populations. The difference between signals from the nuclear and chloroplast genomes can be attributed to pollen dispersal as a result of the biparental inheritance of nuclear microsatellites. Gene flow from pollen can be many times greater than that from seed dispersal, resulting in much lower population differentiation of the biparentally inherited genome under drift-migration equilibrium (Ennos, 1994). A recent study suggests that the importance of seed dispersal may have been underestimated for wind-dispersed species (Bacles et al., 2006), but the patterns of chloroplast and nuclear DNA differentiation in tanoak strongly support a more important pollen-mediated gene flow.

Therefore, the combined patterns of chloroplast and nuclear differentiation can be explained by ancient processes leading to population lineages delimited by the geographic partition of chloroplast haplotypes and subsequent pollen-mediated gene flow leading to relatively low nuclear differentiation. We propose that interior populations of the Sierra Nevada and Klamath Mountains differentiated from coastal populations during the Pleistocene as a result of climatic fluctuations, whereas coastal California populations may have diverged from southern Oregon during more recent climate changes, perhaps during the LGM. We do not have enough evidence yet to determine whether California coastal populations were restricted to few refugia, or multiple refugia. However, the distribution of the coastal haplotype over a very long distance might favor multiple refugia. Expansion of populations following the LGM, coupled with increased gene flow by pollen, are most likely responsible for the relatively low nuclear differentiation. Central California populations are furthest from a Sierran–Klamath corridor that probably explains low levels of admixture in this region.

The phylogeographic pattern that we have detected for tanoak contrasts with that of other Fagaceae in California. The number of chloroplast haplotypes that we detected (six) was few compared with the true oaks of California; 39 haplotypes in *Q. lobata* based upon six cpSSR loci (Grivet et al., 2006, 2008) and 27 haplotypes in *Q. agrifolia* based upon five cpSSR loci (Dodd et al., 2008). Although we cannot directly compare diversity of the chloroplast loci of tanoak with those of *Quercus*, other lines of evidence support a major demographic event in which population extinctions have been important in tanoak. The lack of haplotype admixture in populations is consistent with severe restriction followed by relatively recent range expansion. Assuming loci with comparable mutation rates, contrasting phylogeographic patterns of Californian *Quercus* tree species and tanoak likely stem from two causes: habitat preferences and hybridization. Tanoak is present in a more humid climate, and is replaced by other species on warmer or drier sites (Tappeiner et al., 1990). On the other hand, *Q. lobata* and *Q. agrifolia* are well suited to withstand drought. The dry cool conditions during glacial periods would more likely result in population extinctions in tanoak than in the more drought tolerant true oaks. Hybridization is known to be important in the red oaks of California (Dodd and Afzal-Rafii, 2003) and has been proposed as a means of northward colonization by coast live oak (Dodd et al., 2008). Despite the differential response of oaks and tanoak in California to past climatic change, both groups show a concordant phylogeographic pattern of a high east/west differentiation between the Sierra Nevada and the Coastal Range.

Past demographic change—We used different methods to estimate past demographic change in tanoak from nuclear microsatellite data. Overall, we were unable to detect a signal of population expansion, and the MSVAR analysis found a pronounced long-term decline in effective population size. Pollen records of northern California and southern Oregon show a complex pattern of vegetation change during the Holocene that likely involved major demographic changes in tanoak. Records in coastal northern and central California show that this region was colder and moister during the beginning of the Holocene and that it became considerably warmer and drier after 8000 yr ago (ya), with a peak in aridity around 6000 ya (Adam and West 1983; Rypins et al., 1989; Potito et al., 2006). This increase in aridity would likely have resulted in the restriction of tanoak populations to small patches where microclimatic conditions were favorable. Furthermore, a drying and heating trend has been detected in southern California (Kirby et al., 2007), which could have resulted in a more sustained contraction of the southernmost populations of tanoak. In contrast, the Klamath region and the Sierra Nevada were cold and dry during the early Holocene and became moister after 6500 ya (Mohr et al., 2000

and references therein; Briles et al., 2005; Daniels et al., 2005). Tanoak pollen (as well as most Fagaceae pollen) was completely absent in mid- and high-altitude pollen records in the Klamath region until after 6800 ya, around the same time that precipitation increased in the region (Daniels et al., 2005). The scenario of a warm and arid mid- and late Holocene in coastal California (where we sampled most of our populations) supports our MSVAR and BOTTLENECK results, which detected a range wide long-term contraction and heterozygote excess consistent with a recent bottleneck in the two southernmost sampled tanoak populations, Lompoc and Santa Barbara.

What does the detection time frame of these tests tell us about the timing of possible bottlenecks in tanoak? The BOTTLENECK test is designed to detect severe, short-term reductions in population size. A population bottleneck can be detected with this method before mutation–drift equilibrium is reestablished, approximately $2N_e$ to $4N_e$ generations (Luikart et al., 1998). Generation time in tanoak is about 20 yr (Harlow et al., 1979) and observed population size at Lompoc of about 100–200 individuals. Assuming a bottleneck effective population size of 50–100 individuals, this would suggest that the bottleneck occurred during the last 2000–8000 ya. We estimated global N_e = 404.9 (95% CI: 347.8–478.7) using the Linkage Disequilibrium function in the program NeESTIMATOR (Peel et al., 2004) to estimate the time since population size started changing from the results of the MSVAR analysis. Substituting the values $\log_{10}(t_f)$, and current N_e in the formula $t_f = t_a/N_0$, we obtain an intermediate t_a of 8421 yr [for the mode of $\log10(t_f)$ and the N_e = 404.9] and a maximum of 16 276 yr [for highest value of 95% confidence interval for $\log10(tf)$ and range wide N_e]. This suggests a post-LGM decline in overall populations size, with a modal value coincident with a mid to late Holocene warming in California.

The detection limits of the *k* and *g* tests are relatively long (20 000 yr for a pre-expansion range wide N_e of 1000). However, this long time frame is conditioned on a gradually expanding population that doubles its size every $0.1N_1$ generation (Reich and Goldstein, 1998). This expanding scenario was possible for tanoak populations only for some time during the post-LGM range expansion. However, it is very likely that tanoak's N_e stopped doubling long before it reached its current range because of habitat and resource limitation and that a posterior range contraction took place. Therefore, we conclude that the signal of population expansion in tanoak might be undetectable with the *k* and *g* tests.

Finally, we acknowledge that different factors may bias MSVAR results toward a bottleneck signal including deviations from a strict stepwise mutation model and confounding historical factors (Storz and Beaumont, 2002; Storz et al., 2002). Both of these factors may have played a role in our analysis: multirepeat microsatellite mutations are not uncommon in plants (17% are reported for maize; Vigouroux et al., 2002); and it is unknown how intermixing of previously isolated populations would affect MSVAR estimations. To date, because of its computational intensiveness, there has not been a major assessment of MSVAR's robustness to deviations from the model.

Tanoak conservation—Many coastal tanoak populations have been heavily affected by the SOD epidemic in the last decade; mortality rates of more than 60% have been reported (Maloney et al., 2005) and an exponential increase of mortality has been detected over a 5-yr period (Cobb et al., 2008). Furthermore, the predicted trend for climate warming (IPCC, 2001) is likely to affect tanoak adversely. The loss of tanoak trees throughout the different ecosystems where it is a major component will result in serious consequences for northern California and southern Oregon landscapes; effects could be devastating, including a loss of wildlife that depend on acorns, a raise of fuel loads that would result in more extreme wildfires, and an increase in the risk of soil erosion in coastal ecosystems. Conservation efforts to restore habitat and to preserve the evolutionary potential of this species are likely to be needed in the near future. Our study sheds light on the partition of genetic diversity at putatively neutral loci in both the nuclear and chloroplast genomes. Genetic variation and genetic differentiation throughout the range has to be taken into account to determine conservation priorities and seed sources for long-term preservation and for reforestation efforts.

On the basis of our results for putatively neutral nuclear and cpDNA markers, we identify five main areas for the subdivision of breeding groups: (1) central coastal California (south of the San Francisco Bay area), (2) northern coastal California, (3) the Klamath region, (4) the Sierra Nevada, and (5) southern Oregon. The chloroplast haplotype phylogeny suggests that the Sierra Nevada and Klamath populations form a deeply divergent lineage from coastal populations. Therefore, in any resistance screening and conservation protocols, account should be taken of this biogeographic split. We further identify the southernmost populations near Santa Barbara and Lompoc as having low genetic diversity. These populations are small and levels of inbreeding could be elevated. Further assessment is needed in northwestern California, Oregon, the Klamath, and Sierra Nevada regions to determine if other highly differentiated populations are present and/or to depict secondary contact areas between the different cpDNA haplotype lineages detected in our study. Furthermore, the phylogenetic and population genetic relationship between the tree form and the shrub form [*Lithocarpus densiflorus* subsp. *echinoides* (R. Br. Campst.) Abrams] remain to be unveiled. Preliminary results using the same chloroplast microsatelite markers and sequence region from populations of the shrub form in the Cascade region show two unique cpDNA haplotypes highly differentiated from the geographically adjacent Weaverville population analyzed in this paper (A. Nettel and R. Dodd, unpublished data).

Studies regarding the adaptive genetic variation and, more prominently, variation in susceptibility to the SOD pathogen are crucial for furnishing a comprehensive conservation effort. Concurrent studies concluded that tanoak populations and individual trees express significant differences in susceptibility under common laboratory conditions (K. Hayden and M. Garbelotto, University of California, Berkeley, personal communication). Further studies of the adaptive genetic variation and nonsusceptible genotypes are urgently needed to design a comprehensive conservation strategy in tanoak and to ensure its permanence across northern California and southern Oregon ecosystems where it is present now.

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