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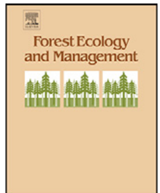

Will all the trees fall? Variable resistance to an introduced forest disease in a highly susceptible host

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ABSTRACT

Although tanoak (*Notholithocarpus densiflorus* syn. *Lithocarpus densiflorus*) is the species most affected by the introduced pathogen *Phytophthora ramorum*, with demonstrable risk of extirpation, little is known about the origin, range or structuring of the tree's susceptibility. We examined variation in resistance to *P. ramorum* using a wound inoculation assay of detached leaves from trees at five geographically separated sites, and a non-wound inoculation assay on twigs from trees at two sites. The structure of variation in resistance was compared to the structure at nine nuclear microsatellite markers.

Resistance varied quantitatively, with 23% and 12% of the variation among individuals and populations, respectively. There was a significant correlation between resistance in detached leaves and lesion size in non-wounding twig inoculations. Among-population genetic diversity at nine microsatellite loci was weakly structured but significantly non-zero, with 9.5% of variation among populations. Within-population neutral genetic diversity was a poor predictor of resistance, and estimates of phenotypic distances for resistance were no greater than neutral genetic distances.

The limited phenotypic and genetic structure we found indicates that tanoaks at all study sites are susceptible, and there is no evidence of prior selection for disease resistance. We conclude that tanoak populations across the species' range are at risk, but local disease dynamics will depend on both host genetics and environmental conditions.

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1. Introduction

Plant populations and communities can be dramatically altered by disease epidemics (reviewed by Burdon et al., 2006; Gilbert, 2002); in particular, introduced forest pathogens have significantly and irreversibly altered the North American landscape (Loo, 2009). In some instances, populations of forest trees have been found to have some degree of resistance to invasive pathogens (reviewed by Sniezko, 2006), as in the cases of white pine blister rust (*Cronartium ribicola*) (Kinloch, 1992) and jarrah dieback (*Phytophthora cinnamomi*) (Stukely and Crane, 1994; Stukely et al., 2007). In other cases, well exemplified by the chestnut blight (*Cryphonectria parasitica*) epidemic, trees have lacked significant resistance to a newly introduced microbe. In California and southern Oregon, ramorum blight and dieback, also known as sudden oak death (causative agent *Phytophthora ramorum*), affects nearly every plant species in the mixed evergreen forest understory and overstory (USDA-APHIS, 2010). The blight was first reported in a single county in California in 1995 (Svihra, 1999) and since that time has spread into 14 California counties and one Oregon county. It has moreover been found in nurseries in the U.S., Canada, and Europe (Canadian Food Inspection Agency, 2010; Rizzo et al., 2002).

Tanoak (*Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon, & Oh, syn. *Lithocarpus densiflorus* (Hook. & Arn.) Rehd.) is the host most susceptible to the ramorum blight pathogen (Davidson et al., 2003; Rizzo and Garbelotto, 2003). Mortality is generally high throughout the infested region and, although the real impact of the disease on this native tree species is not fully understood, there are indications that the epidemic may devastate tanoak populations. Up to 100% plot-level mortality of tanoak stems has been reported in infested stands in the Big Sur region of California (Davis et al., 2010), and McPherson et al. (2010) reported that by 2008, 80% of the tanoaks in plots in Marin County, California that were asymptomatic in 2000 had developed disease symptoms. Tanoak mortality has been reported to be well above baseline levels in infested stands (Maloney et al., 2005; Meentemeyer et al., 2008), and to increase exponentially over time (Cobb et al., 2008). Tanoaks are susceptible at all size classes, in contrast to *Quercus* hosts, which are less susceptible when small (Rizzo et al., 2002). Dead

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stems often resprout from still-living roots; these sprouts are frequently but not necessarily symptomatic of infection, most likely from continued spore production by surrounding hosts (Cobb et al., 2010).

While introduced pathogens are by no means guaranteed to be especially virulent (Desprez-Loustau et al., 2007; Linzer et al., 2009), mass host mortality such as that observed in tanoak is a hallmark of a pathogen host jump to a newly available, naïve host population (André and Day, 2005), as in a recent introduction (Burdon, 1987; Parker and Gilbert, 2004; but see Hansen and Goheen, 2000 for a counter-example). Indeed, *P. ramorum* populations have genetic structures suggestive of a recent introduction via nursery plants. Most of the genetic diversity resides in nursery populations, which have arisen from multiple introductions (Mascheretti et al., 2009) from at least three independently evolving populations (Goss et al., 2009; Ivors et al., 2006). The US wildland populations have arisen from nursery strains (Mascheretti et al., 2008), and consist of only a few closely related, clonally reproducing genotypes of a single mating type with a considerable geographic structure (Ivors et al., 2004, 2006; Prospero et al., 2007). The alternate mating type has been found in US nurseries (Hansen et al., 2003), so the eventual generation of additional *P. ramorum* diversity through sexual recombination is a possibility. Laboratory attempts at crossing the *P. ramorum* mating types have been slow (Brasier and Kirk, 2004; Vercauteren et al., 2010), but recent crosses have produced viable progeny with similar virulence to the parent types (Boutet et al., 2010). The onset of sexual reproduction after the re-introduction of the alternate mating type in the late blight pathogen, *P. infestans*, resulted in more virulent strains and the re-emergence of disease (Goodwin et al., 1998); a similar re-emergence is a valid concern for *P. ramorum*. Cross-species hybridization through either sexual crosses or somatic fusion is another means by which *Phytophthora* may acquire diversity and new host ranges (Érsek and Nagy, 2008); such a hybridization event has been shown to be responsible for a devastating outbreak of disease on *Alnus* trees (Brasier et al., 1999). Two less-virulent but evidently introduced (Linzer et al., 2009) species frequently co-occur with *P. ramorum*: *P. nemorosa* and *P. pseudosyringae* have slightly different environmental limits, but share *P. ramorum's* symptomatology, host range and approximate geographic range (Wickland et al., 2008). The two species' rather distant phylogenetic relationship to *P. ramorum* (Blair et al., 2008) may provide a barrier to their hybridization.

No host-specific structure has been detected in this generalist pathogen (Hüberli et al., 2006a,b; Rosenzweig and Garbelotto, unpublished data), which is believed to spread primarily from infectious propagules originating from foliar hosts, especially California bay laurel (myrtlewood, *Umbellularia californica* (Hook. & Arn.) Nutt.). Tanoak leaf and twig lesions do produce sporangia (Davidson et al., 2008), and there is evidence that the species is more important than *U. californica* for pathogen spread in the very northernmost portion of its range (Hansen et al., 2008).

Despite the recent onset of this particular host-pathogen interaction, some degree of variation in host response is expected. Populations and individuals may vary in their response to a novel pathogen in nonspecific pathways (e.g., in responses to elicitins of non-host responses or in physiological characteristics that limit infection or pathogen spread), or even in specific responses that have evolved in response to other, perhaps related, pathogens (e.g., R-gene responses).[2] A slowing of infection rate in tanoaks despite continued availability of inoculum has been observed after multiple years of monitoring in some sites with early, heavy tanoak mortality, hinting that the surviving trees may possess some forms of disease resistance (McPherson et al., 2010). Moreover, environmental variables such as temperature and moisture can influence both pathogen virulence and host plant resistance responses (Garrett et al., 2006; Zhu et al., 2010). Differences in resistance among populations, whether environmental or genetic in origin, may influence the dynamics of an epidemic across geographic areas (Burdon, 1987).

Tanoaks grow exclusively on the west coast of the US, ranging from Santa Barbara, California, to southern Oregon in the Coast Ranges, and into some portions of the interior Sierra Nevada and Klamath mountain ranges (Fig. 1). It is not valued for timber, but this primary masting species of the redwood forest ecotype supports wildlife and ectomycorrhizal communities (Barrett et al., 2006; Bergemann and Garbelotto, 2006; Massicotte et al., 1999); it is furthermore central to the cultures of Pacific Coast Native American groups (Meyers et al., 2007; Ortiz, 2006). Tanoak genetic structure has only recently been studied. Nettel et al. (2009) reported a low level of differentiation among populations from nuclear microsatellite markers, likely due to historically prolific pollen flow. Maternally inherited chloroplast markers, however, indicated a strong east/west differentiation between Sierra and coastal populations. Tanoak's genetic diversity at the chloroplast DNA level was very low within populations and within regions; most populations presented a single haplotype and the most common haplotype was almost fixed in all the coastal California populations sampled (Nettel et al., 2009).

The current naturalized range of the pathogen in the US is more limited and, while ever-expanding, lies entirely within the tanoak range (Geospatial Innovation Facility, 2008, Fig. 1). Environmental niche models suggest the species' entire geographic range is favorable to the establishment of *P. ramorum* (Kelly et al., 2007). Consequently, tools to predict the potential for tanoaks to survive the introduction of *P. ramorum*–through escape, natural selection or management intervention–are of great value. At any scale, disease outcome depends on each of host resistance, pathogen virulence, and environmental heterogeneity (McNew, 1960). At the landscape and (meta)population scales, the likelihood of tanoak extirpation will thus be mediated by environmental differences among sites and the genetic makeup and structure of each of the host and the pathogen. Of these, variation in tanoak's resistance/susceptibility to ramorum blight is the least clear. The available literature has largely relied on field observations that necessarily confound host, pathogen, environmental, and temporal effects; only preliminary data from seedling studies that followed the study here described have been published (Hayden et al., 2010).

Thus, our aims were to (1) develop a practicable inoculation assay to compare susceptibility of tanoak individuals; (2) obtain a first portrait of variability of tanoak resistance to *P. ramorum*, including its geographic structure and the correlation between leaf and twig symptom development; and (3) determine the genetic structure of the populations surveyed and test whether the structure in resistance correlates with the structure in genetic diversity.

[2] Traditionally, pathologists have categorized pathogen-specific responses as the qualitative, resistance-virulence interactions in Flor (1951) Flor's (1951) classic gene-for-gene model, and nonspecific interactions as partial, quantitatively measured and quantitatively inherited. The latter have sometimes been called "susceptibility". Further characterization of the pathways and genes involved has revealed that the categories are not so neat: single R-genes may confer partial, or quantitatively measured resistance, while qualitative responses may be controlled other than by R-genes, and may even be quantitatively inherited (Bent and Mackey, 2007; Huang, 2001). In this report, we attempt to achieve clarity without making arbitrary distinctions by referring to any reduction in lesion extent as resistance, and to any reduction in infection incidence as infectivity.

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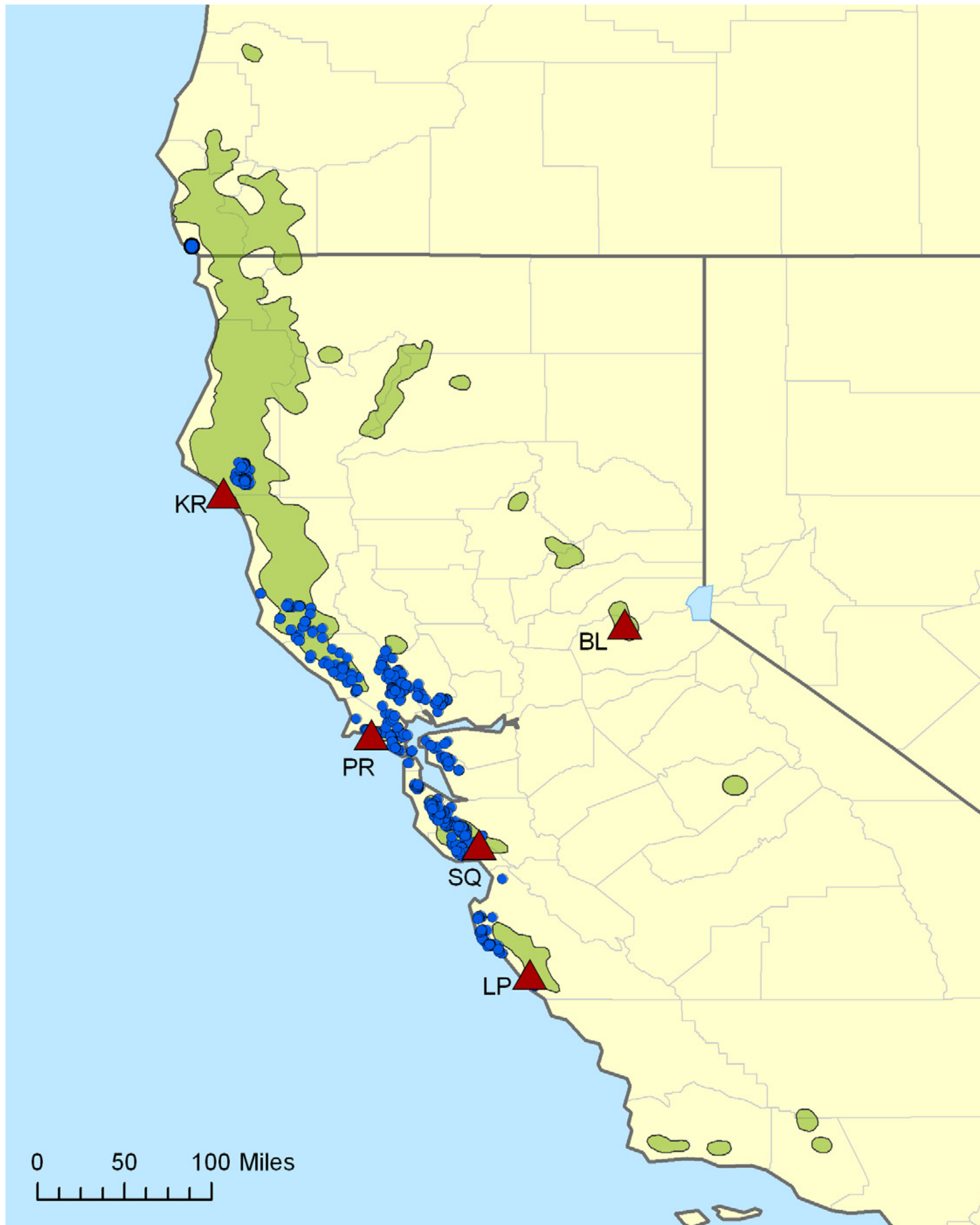

Fig. 1. Location of study populations. Study sites (triangles) are shown in relation to the geographic range of tanoak (*Notholithocarpus densiflorus*) (shaded areas, US Department of the Interior, 2008), and confirmed findings of *Phytophthora ramorum* (circles; California: Geospatial Innovation Facility, 2008; Kelly and Tuxen, 2003; Kelly et al., 2004; Oregon: ODA Plant Division, 2006). The states shown are Oregon to the North, California to the South, and Nevada to the East; the Pacific Ocean lies to the West.

2. Materials and methods

2.1. Field survey: host resistance

We set out to determine how resistance to *P. ramorum* was structured within tanoak's geographic range. For this first portrait, no sources of seedlings from natural populations were available, so phenotypic measurements were taken on detached leaves of adult trees (described in detail below). We sampled five populations within California at the following sites: the Los Padres National Forest, Monterey County (LP); the Soquel Demonstration State Forest, Santa Cruz County (SQ); Point Reyes National Sea Shore, Marin County (PR); the King Range National Conservation Area, Humboldt County (KR); and the University of California Blodgett Forest Research Station, El Dorado County (BL) (Fig. 1, Table 1). Climate data for the four weeks prior to collection were extracted from the nearest available weather station within the microclimate, except for Soquel precipitation data, which were taken by Brook Kraeger at SQ. While both pathogen and host are found further north in coastal Oregon, the sampled sites nearly span the current range of the pathogen in the United States and are a good representation of the geographic range of tanoak. Sites in infested areas were limited to plots that had experienced relatively low mortality from the disease (less than 30%) at the time of sampling and are thus assumed to have been in relatively early stages of the disease.

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Table 1
Locations of the tanoak study populations with climatic data for the month prior to collection, including average daily maximum and minimum temperatures, daily average temperature, and total rainfall.

Site	Location	AveMax (°C)	AveMin (°C)	Ave (°C)	Precip (cm)
KR	40°00N, 120°00W	18.0	11.1	14.6	17.1
BL	38°55′N, 120°39′W	22.3	10.1	16.2	7.7
PR	38°00′N, 122°46′W	20.7	7.9	13.1	12.3
SQ	37°05′N, 121°52′W	21.2	11.4	16.3	7.7
LP	36°00′N, 121°27′W	22.2	8.5	15.3	0.8

2.2. Mycelial inoculation assay

At each site/population (hereafter simply called population), 10 trees per transect were tagged at 10 m intervals on each of three 100 m linear transects. Multiple small branches were sampled from each tree at all populations over a four day period and stored at 12 °C until collections were complete. Ten sub-sampled leaves per tree were assayed with a detached leaf inoculation, whereby a plug of 10% V8 agar containing mycelia from *P. ramorum* isolate Pr75 (provided by D. Rizzo, University of California) was placed on the cut petiole. All inoculations were performed in a single day, in random order. Pr75 was originally isolated from *Quercus agrifolia* Née, and was inoculated and re-isolated from tanoak leaves prior to this experiment, to ensure that the culture had not lost viability while in storage. Isolate Pr75 is of intermediate pathogenicity on both *Q. agrifolia* and *U. californica*, as compared to 44 other *P. ramorum* isolates (Hüberli et al., 2006a,b). The inoculated leaves, plus one sham inoculation per tree, were incubated at 18–20 °C in randomly assigned moist chambers for two weeks. At the end of the incubation period, leaves were digitally scanned, leaf and lesion areas were measured using the software program ASSESS (American Phytopathological Society, Winnipeg, Canada), and leaf pieces were plated on PARP selective media (Erwin and Ribeiro, 1996) to confirm *P. ramorum* infection.

Lesion areas within and among populations were analyzed using JMP v5.0 (SAS Institute, Cary, NC, USA). To determine whether lesion area should be analyzed on its own or as a percentage of leaf area covered (PLAC), a hierarchical mixed model was chosen for each of leaf area and PLAC by backwards elimination, and then the two models (Eq. (1) and (2), below) were compared to each other by likelihood ratio test.

$$y_{ijkl} = \beta_0 + \beta_1 x_{ikj} + \gamma_i + \delta_{ij} + \eta_l + \varepsilon_{ijkl} \quad (1)$$

where y_{ijkl} is the log-transformed lesion area, β_0 and β_1 are parameters to be estimated; x_{ijk} is the log-transformed area of the kth subsampled leaf of the jth tree within the ith population; γ_i, δ_{ij}, and η_l are random effects of the population, the tree within population, and the lth moist chamber; and ε_{ijkl} is residual variation.

$$y_{ijkl} = \beta_0 + \gamma_i + \delta_{ij} + \eta_l + \varepsilon_{ijkl} \quad (2)$$

where y_{ijkl} is the log-transformed PLAC of the kth subsample from the jth tree in the ith population, incubated in the lth chamber, β_0 is a parameter to be estimated; γ_i, δ_{ij}, and η_l are random effects of the population, the tree within population, and the lth moist chamber; and ε_{ijkl} is residual variation. Leaf area is not included as an effect in Eq. (2), because it appears as the denominator of the dependent variable. A Box-Cox transformation was performed to confirm the appropriateness of the log transformation for each of Eqs. (1) and (2).

We calculated the upper bound of broad-sense heritability for lesion area as repeatability,

$$r = \frac{\sigma^2_{\text{tree}}}{\sigma^2_{\text{tree}} + \sigma^2_{\text{chamber}} + \sigma^2_{\text{residual}}} \quad (3)$$

where σ^2 is the variance component of the random factor indicated by each subscript. We assumed measurement error to be negligible. A large value for measurement error would have the effect of increasing our estimate of repeatability, so our assumption is conservative (Lynch and Walsh, 1998; Saether et al., 2007).

Least square means estimates of lesion area for each population (JMP v5.0) were regressed against each of the climatic variables average daily minimum temperature, average daily maximum temperature, average daily temperature, and total precipitation – to discern potential relationships of resistance in detached leaves to the climate in parental populations in the month prior to collection.

2.3. Zoospore inoculation assay

To examine any correlation between a tree's resistance to pathogen spread within its tissue and its resistance to initial infection, we used a non-wounding zoospore inoculation to infect twigs from 14 trees at the SQ and PR sites. These trees were the most and least susceptible individuals from the two most and least susceptible populations in the previous detached leaf assay. We inoculated 20 twigs, each of which appeared to be one year's growth or older, from each tree with a zoospore solution (~10^4 spores/ml) of Pr75 dropped onto the leaf axil, wrapped in a wax film cup (Parafilm M, SPI Supplies). Ten twigs from each tree were incubated at 10 °C and another 10 were incubated at 20 °C. After two weeks, pieces of cambium and bark from the twigs and sections of the leaves' midribs were placed on PARP selective media and monitored for *P. ramorum* growth. Isolations were attempted at 0, 2, and 4 cm from the inoculation point; and from two places on each leaf: the base and the midpoint. Each twig was scored qualitatively, as infected or not infected, and semi-quantitatively, by the maximum distance from the inoculation point of positive isolation. Two trees were removed from the analysis because *P. ramorum* was recovered from sterile water controls, presumably because the twigs were already infected at the time of collection.

Infectivity was modeled with a generalized linear model in the R statistical framework (R Development Core Team, 2009) with the lme4 package (Bates and Maechler, 2009). The following model with a logit-linear link was chosen by iterative deletion:

$$y_{ij} = \beta_0 + \beta_1 x_i + \beta_1 x_j + \varepsilon_{ij} \quad (4)$$

where y_{ij} is the proportion of twigs from which *P. ramorum* could be recovered, β_0, β_1, and β_2 are parameters to be estimated, x_i is the resistance ranking from the previous assay, ranging from 1–12, and x_j is a factor corresponding the incubation temperature. Quasibinomial errors were specified to account for overdispersion.

The correlation of lesion spread within twigs to detached-leaf measures of resistance was analyzed using the same model as for infectivity, except that in this case, the dependent variable y_{ij}, corresponded to the maximum distance from the inoculation point from which *P. ramorum* was isolated within the twig. Distances were measured as discrete integers, so errors were assumed to follow a Poisson distribution and a log-linear link was employed.

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2.4. Field survey: host population structure

To determine the genetic diversity and structure of tanoaks at the five survey populations, DNA was extracted from mature leaves taken from 191 trees, including most of the trees that were sampled for resistance as well as additional trees at most populations. We were unable to recover DNA from 15 previously sampled KR trees because of sample contamination during storage. The number of samples per population ranged from 15 at KR to 57 at SQ. We used a simplified CTAB (cetyltrimethyl ammonium bromide) method (Cullings, 1992) for DNA extraction. Eleven microsatellite loci (LD1, LD3, LD5, LD7, LD8, LD10, LD12, LD13, LD14, LD17, LD19; Morris and Dodd, 2006) were amplified by PCR as described in Nettel et al. (2009), and as described, loci LD13 and LD19 had significant values of homozygote excess consistent with null alleles in at least four of the sampled populations. These loci were excluded from our analyses.

We estimated genetic diversity and within-population inbreeding coefficient (F_{IS}) per population using software FSTAT ver. 2.9.3.2 (Goudet, 2002). The two diversity estimators obtained were Nei (1987) Nei's (1987) gene diversity (H_e) and allelic richness by the rarefaction method (R_t) (El Mousadik and Petit, 1996; Petit et al., 1998). We further estimated the 95% bootstrap confidence interval of allelic richness for each population by analyzing 100 re-sampled populations (alleles re-sampled with replacement). All the re-sampled populations were adjusted to a sample size of 15 because genetic diversity estimators are heavily influenced by the sample size. To study the genetic structure of tanoak, we also estimated the overall and pairwise values of the fixation index, F_{ST} (Weir and Cockerham, 1984), using ARLEQUIN ver. 3.0 (Excoffier et al., 2005). Partition of genetic diversity among and within populations was analyzed by analysis of molecular variance (AMOVA, Excoffier et al., 1992) using ARLEQUIN ver. 3.0.

2.5. Correlation between variation in resistance and neutral genetic markers

We compared neutral genetic structure (F_{ST}) to P_{ST}, the phenotypic approximation of Q_{ST}, the measure of population structure of additive genetic variance in quantitative traits (Spitze, 1993). P_{ST} is problematic in that phenotypic measurements of individuals in natural populations cannot distinguish between environmental and genetic sources of variance. Nonetheless, with sensitivity tests of assumptions of the amount of between- and within-population variance due to additive genetic effects, the relative magnitudes of P_{ST} and F_{ST} may offer insight into the forces driving among-population trait differentiation (Pujol et al., 2008; Saether et al., 2007). We used the Saether et al. (2007) estimation and sensitivity test:

$$P_{ST} = \frac{g\sigma^2_{\text{population}}}{g\sigma^2_{\text{population}} + 2h^2\sigma^2_{\text{within population}}} \quad (5)$$

where g is the proportion of variance among populations due to additive genetic effects, h^2, narrow-sense heritability, is the proportion of variance among individuals within populations contributed by additive genetic effects, $\sigma^2_{\text{population}}$ is the component for among-population variance, and $\sigma^2_{\text{within population}}$ is the sum of the variance components among individual trees and residual variance. P_{ST} was calculated for the entire dataset for $0 \leq g \leq 1$, and $0 \leq h^2 \leq 0.25$. We assumed relatively low values for h^2 because the upper bound for broad-sense heritability, estimated from the repeatability to be 0.27, is necessarily larger than h^2. This sensitivity test indicated $P_{ST} > F_{ST}$ only where $g \geq 0.2$ (Fig. 4 in Appendix A). So, we assumed g and h^2 to be low and equal, and omitted them from further calculations of overall and pairwise P_{ST}, rather than assign them hypothetical values.

Pairwise P_{ST} was compared to F_{ST} between populations both by a paired-t test using JMP and by Mantel tests with the ecodist package in R (Goslee and Urban, 2007). The Mantel tests were of P_{ST} compared to F_{ST} alone, as well as of $P_{ST}/(1-P_{ST})$ to $F_{ST}/(1-F_{ST})$ holding the natural log of geographic distance constant (Saether et al., 2007). In each case the null hypothesis of no correlation was tested using 10,000 iterations.

To understand the relationship between genetic differentiation in tanoak and the observed resistance to *P. ramorum*, we used Mantel tests to test two additional null hypotheses. $H1_0$ is that genetic similarity at the population level is uncorrelated with phenotypic similarity in resistance to *P. ramorum*; $H2_0$ is that there is no correlation between the individual genetic similarity and the individual response to the pathogen.

The first hypothesis was tested by comparing the pairwise F_{ST} matrix with the matrix of pairwise arithmetic differences between population average lesion size. Additionally, we compared the pairwise F_{ST} matrix with the matrix of distances of ranked values of the individual variance in lesion size averaged for each population. For this latter matrix, we first obtained the variance in lesion size per individual and the mean individual variance per population. Then we standardized these values by assigning a rank of 1 to the population with the highest mean (individual) variance and a rank of 0 to the population with the least mean variance; intermediate values were then calculated proportionately. Finally, we performed partial Mantel tests to account for the effect of isolation by distance between populations by adding a third matrix which contained the pairwise geographic distances between populations.

For the second hypothesis we used a matrix of pairwise relatedness among individuals (Wang, 2002), and either (i) a matrix of distances between average individual lesion size, or (ii) a matrix of Euclidean distances of ranked values of the individual variance in lesion size. To account for population-level structure, we also did a partial Mantel test by including a third matrix, which consisted of binary values where 0 corresponds to individuals from different populations and 1 corresponds to individuals from the same population. These Mantel tests were performed with IBDWS ver. 2.00 (Jensen et al., 2005) and significance was assessed by examining 10,000 random permutations for each comparison.

3. Results

3.1. Field survey: host resistance

3.1.1. Mycelial inoculation assay

We found considerable variation in the size of lesions developed by individual trees inoculated with *P. ramorum*. Lesions spread primarily along the midvein, but in contrast to other inoculations of tanoak leaves from nursery-grown plants (Hayden et al., 2010), there was measurable lateral spread into the leaf blade. Lesion area, rather than length, was therefore analyzed as the variable of interest.

There was a significant positive correlation between leaf area and lesion area ($r = 0.66$, $b = 0.61$, SE of the slope = 0.02, $P < 0.0001$). Eq. (1), in which leaf area was included as a covariate, was a significantly better fit to the data than Eq. (2) (Likelihood Ratio Test, LRT = 380, df = 1, $P < 0.0001$). For this reason, and because no lesion covered more than 5% of the leaf, PLAC was rejected as a variable of interest and all estimates of resistance reported here were obtained using Eq. (1).

Twenty-three percent of the variance in lesion area was among individuals, while 12% of the total phenotypic variance resided

Table 2
ANOVA (restricted maximum likelihood) of resistance across survey locations; response is natural log-transformed lesion area (mm^2). Significance of random factors was tested with model reduction in the R statistical platform (R Development Core Team, 2009).

Source	df	Sum of squares	*F* ratio/*L* ratio	Prob > *F*
Population	4	2.86	17.94	<0.0001
Tree (population)	152	34.40	215.13	<0.0001
Ln (leaf area)	1	48.10	714.11	<0.0001
Chamber	37	4.49	1.80	0.0025

Random factor	Variance component	Std. error	95% lower	95% upper	% of total
Population	0.014	0.011	0.005	0.151	12.03
Tree (population)	0.027	0.004	0.020	0.037	23.44
Chamber	0.004	0.001	0.002	0.009	3.61
Residual	0.069				60.92
Total	0.114				100

among populations (Table 2). Repeatability, the upper bound for broad-sense heritability of lesion area, was $r = 0.27$ (95% CI 0.22–0.32).

Lesion areas varied from 6.7 mm^2 to 118.8 mm^2, with individual trees' means ranging from 10.7 mm^2 (SE 0.7 mm^2) to 59.0 mm^2 (SE 10.0 mm^2). Leaf area was significantly associated with lesion area, so leaf area was included in the model, and population resistance rankings are presented as means adjusted for leaf area. Two populations were significantly different from the others after correction for multiple comparisons with Tukey's HSD: PR trees were more susceptible (adjusted mean lesion area 29.4 mm^2, SE 1.04 mm^2), while SQ trees were more resistant (adjusted mean lesion area 21.6 mm^2, SE 1.04 mm^2) (Fig. 2).

We found no observable relationship between population-wide average lesion area and precipitation or mean daily maximum temperature; there was a marginally significant trend towards smaller lesions in populations with higher daily minimum temperatures ($R^2 = 0.75$, $P = 0.06$).

3.2. Zoospore inoculation assay

There was a trend for trees whose leaves were least susceptible to lesion spread to be more resistant to initial infection by *P. ramorum* zoospores, but this relationship was statistically significant only at a false discovery rate of 0.11 (Table 3). Infection was marginally significantly more likely to occur at 20 °C incubation than at 10 °C ($P = 0.04$). Twigs from more resistant trees developed significantly smaller lesions than those from more susceptible individuals regardless of temperature ($P = 0.02$, Table 4), and infection spread was significantly greater at 20 °C in both more and less-susceptible individuals ($P = 0.0002$).

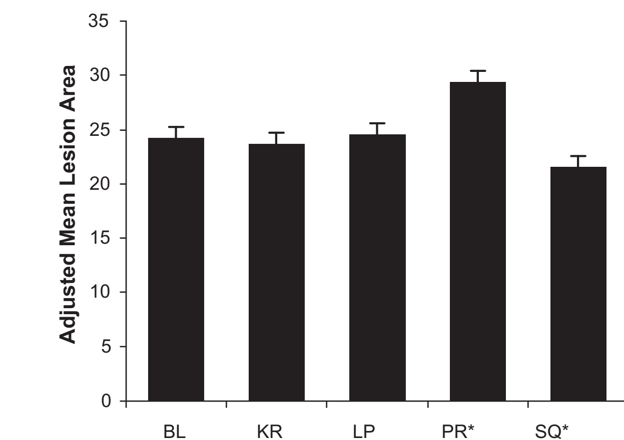

Fig. 2. Mean *Phytophthora ramorum* leaf lesion area (mm^2) in tanoaks at each of five sites, adjusted for leaf area. Data were natural log transformed prior to analysis. Sites with estimates statistically significantly different from the others at $P < 0.05$ are marked with an asterisk. Error bars represent one standard error of the mean.

Table 3
Likelihood ratio effect tests for generalized linear model of the presence/absence of *Phytophthora ramorum* in tanoak twigs after non-wounded inoculation with zoospores. "Resistance" is the ranking of the tree based on the detached leaf inoculation, where 1 was least susceptible.

Source	Estimate	Std. error	*t*-value	*P*
Resistance	0.070	0.043	1.617	0.11
Incubation temperature	0.636	0.303	2.093	0.04

Table 4
Likelihood ratio effect tests for generalized linear model for lesion spread within tanoak twigs, as measured by the maximum distance of positive isolations of *Phytophthora ramorum* after zoospore inoculation, modeled with Poisson errors. "Resistance" is the tree's resistance ranking based on mean lesion area in a detached leaf inoculation.

Source	Estimate	Std. error	*z*-value	*P*
Resistance	0.043	0.019	2.268	0.023
Incubation temperature	0.487	0.133	3.675	0.0002

Table 5
Descriptive statistics for sampled populations of tanoak using nine microsatellite loci: Number of trees sampled (n),) gene diversity (H_e), allelic richness (R_t), and inbreeding coefficient (F_{IS}). Numbers in parenthesis correspond to the standard error.

Population	n	# private alleles	H_e	R_t	F_{IS}
BL	54	10	0.58 (0.02)	3.90 (0.18)	0.133
KR	15	2	0.54 (0.02)	3.37 (0.20)	0.079
LP	32	2	0.46 (0.03)	3.36 (0.20)	0.071
PR	33	4	0.50 (0.02)	3.55 (0.22)	0.113
SQ	57	3	0.46 (0.02)	3.14 (0.20)	0.046
Total	191				

3.3. Field survey: host population structure

The two point estimates of genetic diversity among tanoak populations, H_e and R_t, ranged from 0.46 (LP) to 0.58 (BL) and 3.14 (SQ) to 3.90 (BL), respectively (Table 5). Bootstrap 95% confidence intervals of allelic richness per population overlapped among most populations, except for comparisons between SQ–BL. (Fig. 3). Within population estimates of the inbreeding coefficient, F_{IS}, ranged from 0.05 in SQ to 0.13 in BL. However, none of the estimates was significantly different from zero after correction for multiple comparisons.

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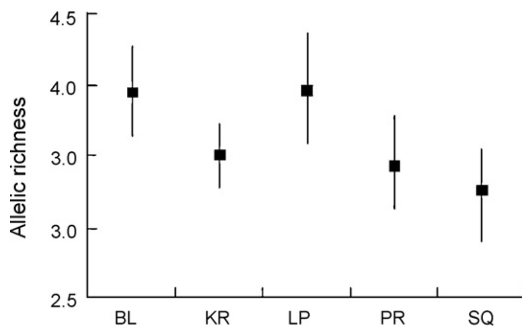

Fig. 3. Plot of tanoak's average allelic richness per population estimated from 9 nuclear microsatellite loci using the rarefaction method. Error bars correspond to the 95% confidence interval estimated from 100 resampled populations.

Table 6
Pairwise estimates of F_{ST} and P_{ST} for the studied tanoak populations. All values of F_{ST} were significantly different from 0 ($P < 0.05$) after correction for multiple comparisons. There was no significant correlation between F_{ST} and P_{ST} by Mantel tests.

	LP	SQ	PR	KR
F_{ST}				
SQ	0.061			
PR	0.127	0.083		
KR	0.128	0.1	0.057	
BL	0.12	0.1	0.093	0.058
P_{ST}				
SQ	0.066			
PR	0.143	0.273		
KR	0.013	0.04	0.179	
BL	0	0.08	0.095	0

Private alleles were detected in all populations, ranging from 2 in KR and LP to 10 in BL. Overall population differentiation, F_{ST}, was 0.097 (95% CI 0.067–0.129). Pairwise F_{ST} estimates ranged from 0.057 between PR and KR to 0.128 between LP and KR. All pairwise F_{ST} values were significantly different from zero ($P < 0.05$, after correction for multiple comparisons, Table 6). Consistent with the species estimate of F_{ST}, the partition of genetic diversity by AMOVA attributed 9.5% ($\Phi_{ST} = 0.095$) of genetic variation to population divergence and 90.5% ($\Phi_{IS} = 0.905$) to intrapopulation diversity (Table 7).

3.4. Correlation between variation in resistance and neutral genetic markers

Overall P_{ST} was 0.100 (95% CI 0.063–0.138), almost exactly overlapping the confidence interval for the estimate of overall F_{ST}. P_{ST} was greater than F_{ST} at the two populations with significantly different estimates of disease resistance, SQ and PR, (pairwise $P_{ST} = 0.18$, pairwise $F_{ST} = 0.08$, Table 6), but there was no overall trend towards greater phenotypic than neutral genetic differentiation ($t = -0.12$, $P = 0.90$). Mantel tests on all pairwise comparisons further revealed no significant correlation of P_{ST} and F_{ST}, even with geographic distance held constant (P_{ST}–F_{ST}, $r = -0.22$, $P = 0.51$; P_{ST}–F_{ST} + geographic distance, partial $r = 0.14$, $P = 0.70$).

Table 7
Analysis of molecular variance (AMOVA) of the sampled tanoak populations based on microsatellite markers.

	df	Variance component	% of total variance	P
Among populations	4	0.23	9.55	<0.001
Within populations	395	2.19	90.45	<0.001

Mantel tests further revealed no significant correlations between genetic distances/coefficients of relatedness and similarities in phenotypic response to the pathogen at the population or individual level. Mantel r values for these correlations were less than $r = 0.08$ (results not shown). Correlation between geographic distance and genetic distance as a single-effect model was also non-significant; thus isolation by distance between the sampled tanoak populations was rejected (Mantel $r = 0.23$, $P = 0.23$).

4. Discussion

We describe here the first application a new assay to survey resistance to a devastating disease in tanoak, a key species in western US coastal evergreen forests. In order to experimentally assess variation in host resistance, a suitable assay must first be developed. The choice of assay to test for resistance is notoriously problematic: disease resistance may result from a variety of different traits, and an individual judged resistant by one assay may easily be susceptible by another (e.g., Kinloch et al., 2008; Oh et al., 2006). Each assay may nevertheless provide information about host fitness, despite apparent conflicts (Kinloch et al., 2008; Sniezko, 2006). Tanoak trees are unique among known hosts of *P. ramorum* in that they regularly develop leaf, twig, and bole symptoms; *P. ramorum* acts as a trunk canker only in *Quercus* hosts, and as a leaf and/or twig blight in all others (Davidson et al., 2003). Trunk cankers confer the high host mortality commonly associated with the pathogen, but pathogen sporulation occurs on leaves and twigs (Rizzo et al., 2003; Davidson et al., 2008). Inoculations of entire trees were neither feasible nor desirable in this study, because of the risk for pathogen escape. Under-bark inoculations of branches detached from *Quercus* species have been used to assess variation in resistance to ramorum blight (Dodd et al., 2005; Dodd et al., 2008b; Hüberli et al., 2006a), but in tanoaks this method frequently yields nearly invisible lesions and large measurement error, precluding its use on a large scale (Hayden and Garbelotto, unpublished data). Detached-leaf inoculations with zoospore suspensions, both wounding and nonwounding, have been used to gauge host resistance to *P. ramorum* in its foliar hosts (Anacker et al., 2008; De Dobbelaere et al., 2010; Grünwald et al., 2008; Hüberli et al., 2006a). But tanoak leaves, again, often develop asymptomatic infections from zoospore solutions (Hansen et al., 2005).

The wounding, detached-leaf mycelial inoculation developed in this study resulted in clearly visible lesions, could be performed rapidly enough that all of the samples were assayed at the same time, and resulted in lesions that spread primarily on the midrib and so resembled tanoak leaf lesions that are often associated with dieback. Moreover, results from the detached-leaf mycelial assay were positively correlated with lesion sizes in woody tissue inoculated with a more labor-intensive non-wounding technique using zoospores (Table 4, $P = 0.03$). Thus, the leaf inoculation may be used to assay the variable most likely to influence tanoak survival on infection with *P. ramorum*: its ability to contain the spread of the pathogen within its tissue. If the possible correlation between rates of spread of the pathogen and rates of infection by zoospores (Table 3, $P = 0.11$) were to be confirmed – independently of whether these traits are genetic or environmental in origin – trees identified as resistant to pathogen colonization within their tissues may be also subject to lower levels of infection from the start. It is notable that infection rates were nonzero in all cases. If complete, qualitative resistance to ramorum blight infection exists in tanoak, it is so rare that it has not been observed.

Using the mycelial inoculation on leaves detached from trees in wild populations, we found small, statistically significant differences in quantitative resistance to ramorum blight among trees and populations. Most of the variance resided within individuals, and

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repeatability, the upper bound for broad-sense heritability (Lynch and Walsh, 1998), was 0.27 (95% CI 0.22–0.32). Although the design of this study does not allow us to parse genetic from environmental components of phenotypic variance, this low value, which is in line with the single-inoculation values reported for a detached-branch *P. ramorum* inoculation of *Q. agrifolia* (Dodd et al., 2008b), indicates a low relative contribution of genetic effects to the measured disease resistance. We infer from these findings a high susceptibility overall. Nevertheless, our upper bound for heritability of $r = 0.27$ is not out of the range that might be important from an evolutionary or management perspective. Most quantitative traits targeted for tree breeding have a narrow-sense heritability – the proportion of phenotypic variance due only to additive genetic effects – in the range of 0.10–0.30 (Carson and Carson, 1989). A common-garden study of Loblolly pine (*Pinus taeda* L.) rooted cuttings for quantitative measures of resistance to fusiform rust (*Cronartium quercuum* f. sp. *fusiforme*) and pine pitch canker (*Fusarium circinatum*) reported broad-sense heritability estimates similar to our upper bound, ranging from 0.19 to 0.50 for fusiform rust and 0.37 to 0.42 for pitch canker, and subsequently estimated narrow-sense heritability at 0.10 to 0.25 and 0.27 for fusiform rust and pitch canker respectively (Kayihan et al., 2005).

These findings are important firstly because they indicate that all of the tanoak populations in our study are at risk. Further, they may be used to guide future resistance screening efforts, for example by placing more emphasis on sampling more trees within populations rather than on expanding the number of populations, given the same amount of sampling effort. Finally, they may be used to generate testable models of disease dynamics based on the small but significant differences we observed among populations. For example, we expect the disease to move faster and take a heavier toll on tanoaks at PR – the most susceptible site we surveyed (and where the pathogen has only recently been observed) – than at SQ, the least susceptible, where the pathogen has persisted at low levels since at least 2001. Anecdotally, this seems to be the case. Alternately, the most susceptible genotypes at SQ may have simply been killed early in the epidemic, leading to a prediction of site-wide increase in ramorum blight resistance over time.

The phenotypic differences we observed are likely derived from a combination of genetic differences separate from those detected by neutral markers and from environmental factors. The environment must be considered first because, out of necessity, this first study of tanoak variation was performed on standing trees in their home populations, rather than on trees raised in a common environment. The detached-leaf assays used to gage resistance were performed at the same time and under the same conditions, and the cut branches from which they were taken had been held in the same conditions for at least 24 h prior. Some physiological effects of climate and/or nutrition nevertheless would be expected to have persisted over the time required for harvest and transport to the experimental laboratory, and we did in fact find a marginally significant trend towards smaller lesions in sites with higher daily minimum temperatures ($R^2 = 0.75$, $P = 0.06$). We found no observable relationship between site-wide measured resistance and precipitation or mean daily maximum temperature. After non-wound zoospore inoculations of twigs, infection rate and lesion spread were both significantly reduced when incubation occurred at 10 °C compared to 20 °C. Tanoak mortality from ramorum blight has been associated with cooler and moister microclimates (Cobb et al., 2008; Davidson et al., 2008; Davis et al., 2010). Our findings suggest that the increased mortality may be as much an effect of host physiology as of pathogen growth requirements: our range-wide leaf assays were all incubated at *P. ramorum*'s ideal growth temperature. Our data further demonstrate that very low temperatures have a counteracting, inhibitory effect on the pathogen. Thus, there is a trade-off in the climatic conditions yielding maximum disease: colder climates lead to higher tanoak susceptibility, but if they are too cold pathogen growth is directly restricted.

We next considered the correlation of neutral genetic structure with the observed phenotypic structure. As with phenotypic variation, there were small but significant differences in neutral genetic variation between populations, with more genetic variation within populations than among populations. No clones were found in the study reported here, indicating that the 10 m spacing between sampled trees was sufficient to avoid re-sampling the same genets. The populations with the highest and the lowest susceptibilities, PR and SQ, had similar levels of genetic diversity, and their 95% CI of allelic richness overlapped. The population with the highest genetic diversity, BL, with $R_t = 3.90$ (SE 0.18) and $H_e = 0.58$ (SE 0.02), showed average resistance. BL, the only inland, Sierra population sampled in this study, was also the most isolated, due to the gap in the distribution range of this tree species caused by the valleys located between the interior and the coastal mountain ranges of California. Interestingly, although BL was characterized by the largest number of private alleles, its levels of differentiation in nuclear markers from other populations (pairwise F_{ST}) were not especially high overall. In general, these findings of moderate levels of genetic diversity and low population genetic structure are consistent with those for the species range as a whole (Nettel et al., 2009), and with expectations for a woody species with a regionally-dispersed geographic range, outcrossing breeding system, wind-dispersed pollen and limited, animal-ingested seed dispersal (Hamrick et al., 1992). They are likewise similar to recent studies of the true oaks in California (Dodd et al., 2008a; Grivet et al., 2008; but see Dutech et al., 2005).

We conclude that tanoak's intrapopulation genetic diversity as estimated from the microsatellite markers is a poor predictor of resistance. Further, Mantel tests showed no relationship between genetic distance and similarity in resistance – implying that none of our relatively few markers were closely linked to loci with a large effect on resistance. Dodd et al. (2005) did not find a relationship between any AFLP markers (whose greater number alone makes finding links more likely) or genetic distance between populations and resistance to ramorum blight in *Q. agrifolia*. Anacker et al. (2008) did find AFLP markers associated with variation in susceptibility in *U. californica*; nevertheless, in the same study the authors inferred environment to have an even greater effect. Among-population differentiation was not significantly different in observed quantitative disease resistance (P_{ST}) than in neutral genetic markers (F_{ST}), so we cannot reject the null model that populations are not differentiated in phenotype beyond random variation (Felsenstein, 1986; Lande, 1992; Spitze, 1993). Environmental influences could have masked true variation, but P_{ST} estimated from wild populations is unlikely to be less than the "true" Q_{ST} (Leinonen et al., 2008). While drawing conclusions about evolutionary forces on traits from phenotypic observations alone is problematic (Pujol et al., 2008), this preliminary investigation finds no indication that these populations had previously experienced different degrees of selection for resistance.

This first study of tanoak resistance structure focused on mature trees in their natural environment; a common garden study is currently underway to better estimate the narrow-sense heritability of resistance in tanoak, and a field study is in progress to determine the effect of the resistance as measured by leaf assays on tree survivorship (Hayden et al., 2010). These studies will allow us to parse genetic from environmental components of variance in resistance, a vital component of either management for resistance through breeding or of disease progress prediction. Additionally, they will allow a more accurate calculation of Q_{ST}, as well as the comparison of the measure across multiple traits, including additional measures of resistance. Increased sampling is also called for: rare alleles responsible for greater quantitative or qualitative resistance may be present elsewhere in the population. In

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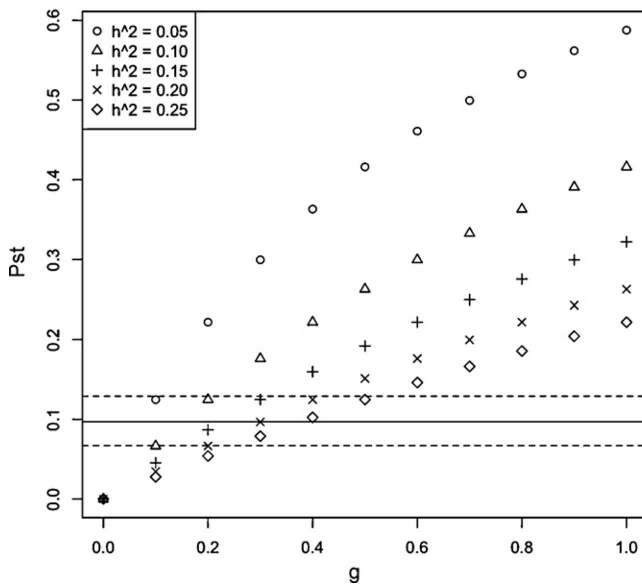

Fig. 4. Sensitivity plot for estimates of P_{ST} of lesion area across all five study populations, with different values of narrow-sense heritability, h^2, and the among-population proportion of genetic variance, g. The solid horizontal line is the estimate of F_{ST} based on neutral markers, with confidence intervals in dashed lines. When g is low and h^2 approaches its upper bound based on repeatability estimates, P_{ST} and F_{ST} converge.

particular, the lack of evidence for prior selection for resistance would imply that any resistance genes should occur at low frequency.

The work we describe here forms a foundation on which a predictive framework for the potential for tanoak populations to evolve in response to the *P. ramorum* epidemic may be built. Our estimates suggest that heritability of resistance is likely to be quite low and accordingly that environmental effects play a significant role, both directly on the pathogen and indirectly by altering the host physiology. This indirect effect has already been documented for coast live oak affected by ramorum blight (Dodd et al., 2008b), and may demonstrably affect the course of the epidemic. The small but significant differences we found in resistance among populations, whether environmental or genetic in origin, provide an important tool for predicting the pace of the epidemic at different sites. We found tanoak populations to be significantly but weakly genetically structured, indicating high gene flow. Gene flow may act to swamp adaptation in populations under strong pathogen pressure by influx from pathogen-free or low mortality populations; this effect will be amplified if the areas of infestation are small relative to the total range of the tree. Conversely, migration may allow marginal populations to persist long enough to adapt by demographically rescuing a declining population and/or increasing genetic diversity, increasing the chances of a novel, more fit gene combination to arise (Forde et al., 2004; Garant et al., 2007; Gomulkiewicz et al., 2000; Lenormand, 2002; Nuismer et al., 2003). Moreover, the trees sprout prolifically from root crowns (Tappeiner and McDonald, 1984), even when the primary stem has been killed by *P. ramorum* (Cobb et al., 2010). Sprouting may allow tanoaks to persist as an understory species for some time, even if all overstory stems have died. Competition from sprouts can limit seedling recruitment, however (Bond and Midgley, 2001). So, in the presence of seed-producing, resistant adults, the persistence of susceptible genotypes' sprouts may ultimately slow evolution.

5. Conclusions

We describe here inoculation assays that may be used to guage at least one form of tanoak resistance to *P. ramorum*. Using a wounding mycelial inoculation assay, the results of which were correlated with those of a non-wounding zoospore inoculation, we found that disease resistance varied primarily within populations but that there was significant variance among populations, echoing the variance structure of microsatellite markers. Although our results indicate that the genetic markers we used were not linked to those controlling the phenotypic variation we observed, and a common garden study will be necessary to parse environmental effects from genetic influences, we have gained a preliminary understanding of the relative role of each. This information will be useful both to guide future sampling schemes and studies, and to allow prediction of the severity of disease based not just on climatic conditions and the presence of tanoaks, but on the average susceptibility of each tanoak population.

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Appendix A.

See Fig. 4.

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