Susceptibility to *Phytophthora ramorum* in a key infectious host: landscape variation in host genotype, host phenotype, and environmental factors

Brian L. Anacker[1,2,4], Nathan E. Rank[2], Daniel Hüberli[3], Matteo Garbelotto[3], Sarah Gordon[2], Tami Harnik[3], Richard Whitkus[2] and Ross Meentemeyer[1]

[1]Department of Geography & Earth Sciences, University of North Carolina, Charlotte, Colvard 5062, Charlotte, NC 28223, USA; [2]Department of Biology, Sonoma State University, Rohnert Park, CA 94928, USA; [3]Department of Environmental Science Policy & Management, 137 Mulford Hall, University of California, Berkeley, CA 94720, USA; [4]Present address: Department of Environmental Science and Policy, 1 Shields Avenue, University of California, Davis, CA 95616, USA

Author for correspondence:
Brian L. Anacker
Tel: +1 530 752 3940
Fax: +1 530 752 3350
Email: blanacker@ucdavis.edu

Received: *6 July 2007*
Accepted: *28 September 2007*

Summary

- Sudden oak death is an emerging forest disease caused by the invasive pathogen *Phytophthora ramorum*. Genetic and environmental factors affecting susceptibility to *P. ramorum* in the key inoculum-producing host tree *Umbellularia californica* (bay laurel) were examined across a heterogeneous landscape in California, USA.
- Laboratory susceptibility trials were conducted on detached leaves and assessed field disease levels for 97 host trees from 12 225-m^2 plots. Genotype and phenotype characteristics were assessed for each tree. Effects of plot-level environmental conditions (understory microclimate, amount of solar radiation and topographic moisture potential) on disease expression were also evaluated.
- Susceptibility varied significantly among *U. californica* trees, with a fivefold difference in leaf lesion size. Lesion size was positively related to leaf area, but not to other phenotypic traits or to field disease level. Genetic diversity was structured at three spatial scales, but primarily among individuals within plots. Lesion size was significantly related to amplified fragment length polymorphism (AFLP) markers, but local environment explained most variation in field disease level.
- Thus, substantial genetic variation in susceptibility to *P. ramorum* occurs in its principal foliar host *U. californica*, but local environment mediates expression of susceptibility in nature.

Key words: amplified fragment length polymorphism (AFLP), disease susceptibility, landscape epidemiology, oomycete, plant–pathogen interaction, sporangia, sudden oak death, *Umbellularia californica*.

New Phytologist (2008) **177**: 756–766

doi: 10.1111/j.1469-8137.2007.02297.x

Introduction

The establishment and spread of infectious plant diseases in natural areas causes dramatic environmental impacts world-wide (Daszak *et al.*, 2000; Anderson *et al.*, 2004). A key challenge in disease ecology and epidemiology is to determine how host–pathogen interactions are formed and mediated in heterogeneous natural systems (Burdon *et al.*, 1989; Carlsson-Graner & Thrall, 2002; Meentemeyer *et al.*, in press a). In particular, knowledge of the mechanisms underlying host susceptibility is needed to increase our predictive understanding of disease dynamics. The genetic background often plays an

important role in determining the susceptibility of a host and, in turn, disease severity (Alexander *et al.*, 1993; Antonovics & Thrall, 1994; Frank, 1997). To date, studies of host plant genetics have largely relied on controlled experiments in glasshouses or experimental field populations. Fewer studies have pursued extensive, direct genotyping of host individuals in natural settings in which a pathogen is spreading, despite the importance of the interaction between genetics and the environment in understanding plant–pathogen dynamics (Alexander *et al.*, 1996; Thrall *et al.*, 2001). Landscape-scale approaches for identifying patterns of susceptibility may be especially useful in environments characterized by significant spatial heterogeneity of habit conditions (Holdenrieder *et al.*, 2004; Ostfeld *et al.*, 2005).

In this study, we examine the degree to which genotypic, phenotypic, and abiotic factors influence susceptibility to the invasive pathogen causing sudden oak death in a key infectious host across an environmentally heterogeneous landscape (Rizzo & Garbelotto, 2003). Sudden oak death is a forest disease responsible for the death of hundreds of thousands of native *Quercus* spp. L. (oak, Fagaceae) and *Lithocarpus densiflorus* (Hook. & Arn.) Rehd. (tanoak, Fagaceae) trees in coastal woodlands of California (Rizzo *et al.*, 2005). This disease is caused by *Phytophthora ramorum* Werres *et al.*, an oomycete (Kingdom: Stramenopila) that produces two types of infections on host species: lethal stem and trunk cankers on several *Quercus* spp. and nonlethal foliar infections on dozens of native plant species in North America and Europe. We focus here on susceptibility of the infectious foliar host *Umbellularia californica* (Hood. & Arn.) Nutt. (bay laurel, Lauraceae) because it is the largest producer of *P. ramorum* inoculum in coastal forests of California (Davidson *et al.*, 2005) and its presence is positively associated with oak mortality (Kelly & Meentemeyer, 2002; Swiecki & Bernhardt, 2002; Swiecki, in press). The role of *U. californica* in the sudden oak death pathosystem is especially significant because of its widespread abundance in California and frequent co-occurrence with vulnerable oak and tanoak host species (Rizzo *et al.*, 2005).

Infection of *U. californica* in nature is patchy at both landscape (Condeso & Meentemeyer, 2007) and regional (Meentmeyer *et al.*, in press b) spatial scales, suggesting a possible genetic basis for susceptibility. We hypothesize that landscape variation in susceptibility of *U. californica* contributes to the heterogeneous distribution of disease observed in nature. To test this prediction, we examined the degree to which susceptibility of the infectious host *U. californica* to *P. ramorum* is related to plant genotype, plant phenotype, and local environment across an environmentally heterogeneous landscape. We ask: how does susceptibility to *P. ramorum* vary among *U. californica* individuals and *P. ramorum* isolates; are *U. californica* phenotype and genotype related to susceptibility; which of these factors influence disease expression in nature? An understanding of host–pathogen and genotype–environment interactions is critical for developing epidemiological models of disease spread (Meentemeyer *et al.*, 2004) and devising management strategies to minimize further impacts of this pathogen.

Materials and Methods

Study sites

We sampled leaves and assessed disease from 12 plots in April of 2005 from three localities (Annadel State Park, Fairfield Osborn Preserve, and Sugarloaf Ridge State Park) located north of San Francisco, California, in Sonoma County (Fig. 1). These plots were selected from a larger network covering a 275-km^2 area established to study the transmission and ecological impact of *P. ramorum*. Upon plot establishment, the diameter at breast height (DBH) of each tree species was measured for all stems that exceeded 5 cm in DBH and 1.4 m in height. For this study, we selected plots that had 10 or more *U. californica* trees and where *P. ramorum* had been demonstrated to occur using standard culturing methods in the laboratory. To minimize potential confounding effects of seasonality on disease expression (Dodd *et al.*, 2005; Meshriy *et al.*, 2006), we chose plots that could be sampled in a single day.

From each of 12 15 × 15 m field plots, we selected up to 10 *U. californica* trees for the detached leaf assay of susceptibility (Table 1). We also compared our plots in Sonoma County to a 13th reference plot in China Camp State Park in Marin County known for high levels of disease expression and oak mortality (Hüberli *et al.*, 2006). Genetic and abiotic data were not collected for the China Camp plot.

Variation among individuals in susceptibility

Sampling and inoculation On 8 April 2005, we collected five branch cuttings (~40 cm in length) from every *U. californica* tree in each of the 13 plots (Table 1). Branches were selected if they had at least 20 asymptomatic leaves (Table 1). The freshly cut ends of branches were placed into water and kept cool until inoculation in the laboratory 3 d later. During development of our leaf inoculation procedure, we found that inoculation of leaves up to 4 d after collection from trees does not affect the resulting lesion area ($P = 0.98$, $n = 13$–18; D. Hüberli *et al.*, unpublished data). In the laboratory, 14 mature, asymptomatic and undamaged leaves were removed from five branches of each tree. Leaf surfaces were surface decontaminated with a 70% ethanol solution and placed into 50-ml tubes containing 300 µl of *P. ramorum* zoospores (2×10^4 zoospores ml^{-1}). Leaf position was standardized so a constant leaf tip area was submerged in each tube (7–8 mm). One control leaf per tree received 300 µl of sterile deionized water, for a total of 102 control leaves. Tubes were incubated at 19°C in the dark in humid chambers overnight. The following day, leaves were placed onto moist paper towels in humid chambers and incubated at 19°C for 8 d. The *P. ramorum* isolate, Pr52 (Centraal Bureau voor Schimmelcultures

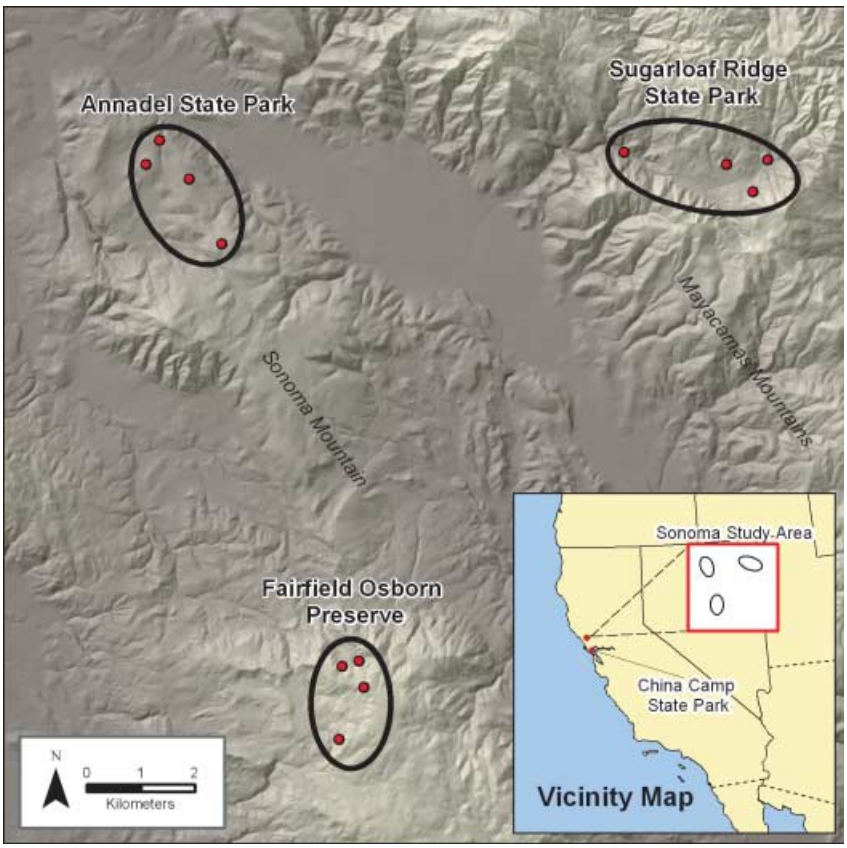

Fig. 1 Locations of study plots in Sonoma County, California, USA, used in the *Umbellularia californica* inoculation and disease assessment study. The plot from China Camp State Park is located ~45 km south of the Sonoma study area. The elevation depicted ranges from 33 to 834 m. See Table 1 for details of the sample design.

(CBS)110537; American Type Culture Collection (ATCC) MYA-2436), was used in all inoculations because it has been shown to be highly pathogenic in previous inoculation studies with *U. californica* (Hüberli *et al.*, 2006). Zoospores were produced for use in inoculations as described by Hüberli *et al.* (2003). Fourteen additional leaves from each of nine to ten trees for one plot per locality ($n = 22$ trees) were inoculated with a local isolate (SSU2-1) recovered from a symptomatic *U. californica* leaf collected in Annadel State Park during March 2005 (Table 1).

Quantification of lesion size Inoculated leaves were scanned on a blue background and the lesion area was quantified using image analysis software (ASSESS, version 1.01; American Phytopathological Society Press, St Paul, MN, USA). To verify that lesions were caused by *P. ramorum*, five randomly selected leaves per tree were plated onto pimaricin-ampicillin-rifampicin-pentachloronitrobenzine (PCNB) agar (P_{10}ARP) containing 25 mg of PCNB, a *Phytophthora* selective agar medium (Erwin & Ribeiro, 1996). In addition, all control leaves were plated, regardless of the occurrence of leaf lesions. To relate lesion area to abundance of *P. ramorum* spores, we quantified *P. ramorum* sporangia and chlamydospores produced following inoculation with Pr52 on seven to ten randomly selected leaves from each of nine to ten trees for one plot per locality ($n = 22$ trees) (Table 1). Sporangia contain mobile zoospores which are released upon landing on a suitable host surface; chlamydospores are resting structures that may allow the pathogen to survive harsh conditions (Rizzo *et al.*, 2002). Sampling leaves for sporangia involved gently scraping the lesion area or region of inoculation, before scanning and plating leaves, with a flat spatula on both leaf surfaces into individual wells containing 300 µl of sterile deionized water of a 24-well microliter plate. The number of sporangia per well was counted, and the presence of chlamydospores noted, after the addition of one drop of phenol-cotton blue.

Phenotypic and genotypic correlates of susceptibility

Quantification of phenotypic traits Five phenotypic leaf traits were recorded, including leaf length and area (measured during the leaf scanning described in the previous section), tree DBH (measured during plot establishment), leaf water content, and leaf toughness. Leaf water content was measured as the difference between fresh and dried weight (g) of five leaves per tree. Leaf toughness was assessed as the average amount of pressure required to puncture a hole in two places (midway along the leaf between the midrib and the margin on both sides) in each of five leaves per tree using a hand-held penetrometer (area 5.5 mm^2; values in kPa mm^{-1}) (Kearsley & Whitham, 1989).

Table 1 Location and tree sample size for 13 plots used in the study

Plot ID	No. of leaves assayed*			Total no. of leaves assayed
	No. of trees sampled	Pr52 isolate	SSU2-1 isolate	
Sonoma County				
Annadel State Park				
1	7	96 (13.7)	97 (13.9)	193
4	9	100 (11.1)	–	100
5	8	103 (12.9)	–	103
15	10	133 (13.3)	–	133
Fairfield Osborn Preserve				
1	8	84 (10.5)	–	84
2	9	123 (13.7)	–	123
3	5	62 (12.4)	–	62
5	7	97 (13.9)	94 (13.4)	191
Sugarloaf Ridge State Park				
13	8	94 (11.8)	96 (12.0)	190
15	8	111 (13.9)	–	111
16	9	110 (12.2)	–	110
19	9	124 (13.8)	–	124
Marin County				
China Camp State Park				
1	5	69 (13.8)	–	69
Totals				
–	102	1306	287	1593

*No. of leaves assayed per tree (mean no. per tree).
The *Umbellularia californica* leaf assay consisted of scoring leaf area, length, and width and resulting lesion area. Sonoma County leaves were also scored for leaf toughness and water content.

Quantification of genotypic traits – amplified fragment length polymorphism (AFLP) analysis Leaf samples were collected in the field from June to mid-August 2004 for molecular analysis using AFLP markers. For each tree, two asymptomatic leaves were collected, wiped clean with a dilute bleach solution, and placed into plastic bags containing silica granules for immediate desiccation. DNA extractions were performed using the Qiagen DNeasy High-Throughput Plant Kit (Qiagen, Valencia, CA, USA). AFLP procedures (Vos *et al.*, 1995) were followed as described in the LiCor AFLP Kit for Large Plant Genome Analysis (LiCor, Lincoln, NE, USA). *Eco*RI and *Mse*I primers for selective amplification were combined as follows: *Eco*RI-ACT/*Mse*I-CAA (coded as bb), *Eco*RI-AAG/*Mse*I-CTC (coded as g), and *Eco*RI-ACC/*Mse*I-CTA (coded as y). Electrophoresis was performed on the Applied Biosytems 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Fragments of between 80 and 550 bp were sized and scored using GeneMapper software (Applied Biosystems). Putative loci were named with the one or two letter prefix given for the primer pairs (as above) and a number representing fragment size in base pairs (Gordon, 2006).

Factors influencing disease expression in nature

Quantification of symptomatic leaves During the period of peak disease expression in 2005 (24 March to 19 April), we visited each plot to quantify *P. ramorum* infection level on *U. californica*. We assessed infection level by counting the number of symptomatic leaves on each *U. californica* individual for 60 s. Timed sampling was performed because of the impossibility of completely censusing all symptomatic leaves on dozens of trees. Also, the patchy nature of infection within trees would make it very difficult to select a representative sample of individual branches for complete censusing. The timed sampling technique has been shown to be highly repeatable across trained observers and to not differ significantly across time intervals of 60, 90 and 120 s (see Condeso & Meentemeyer, 2007). Twelve leaves were collected from each tree and plated on P_{10}ARP to confirm the presence of *P. ramorum* in the counted leaves.

Quantification of environmental variation To characterize environmental differences among plots that may influence *U. californica* susceptibility and *P. ramorum* activity, we recorded hourly measurements of understory temperature and relative humidity with a microclimate data logger (model H08-032-08; Onset Corporation, Bourne, MA, USA) housed in a solar radiation shield 1 m off the ground in each plot. We calculated mean daily minimum, mean and maximum temperatures during the 2004–2005 rainy season (1 November to 1 May). Precipitation data from the same season were also collected from 15 rain gauge loggers (model RG2; Onset Corporation)

across the study area. For each plot location, cumulative daily precipitation was spatially interpolated using geostatistical co-kriging methods (Condeso & Meentemeyer, 2007). Finally, we calculated topographic variables for each plot location using a United States Geological Survey (USGS) 10-m digital elevation model in a Geographic Information System (GIS): elevation, average potential direct beam solar insolation (SII) between December and May (Dubayah, 1994), and topographic moisture index (TMI), a measurement of soil moisture based on local topography (Beven & Kirkby, 1979; Moore *et al.*, 1991).

Statistical analysis

Unless otherwise stated, all statistical analyses were performed using JMP 5.1 (SAS Institute, Cary, NC, USA).

To assess the spatial structure of variability in lesion response to inoculation, we used an ANOVA of lesion area with individual, plot and locality as random effects. To compare lesion response at a broader county scale, we used ANOVA with mean lesion area response per tree between the two sites (Sonoma and Marin). To determine the relationship between lesion area and sporangia counts, we performed an analysis of covariance (ANCOVA) with tree as a random grouping factor and lesion area as the continuous independent variable. We also determined whether trees differed with respect to the relationship between lesion area and sporangia production by adding the covariate by grouping factor interaction term to the statistical model. We analyzed the relationship between lesion area and presence or absence of chlamydospore count using a generalized linear model with the same grouping factor and covariate as in the ANCOVA, and the chlamydospore status as an ordinal response variable. To compare *U. californica* susceptibilities to different isolates of *P. ramorum*, we used linear regression analysis of mean lesion area response to isolate Pr52 and lesion area response to isolate SSU2-1.

Correlations between lesion area response and phenotypic traits taken together were analyzed using a best subset regression, with mean lesion area per tree as the dependent variable and mean leaf water content, leaf length, leaf width, leaf area, mean leaf toughness, and DBH per tree as independent variables. Interaction terms among phenotypic variables were also tested as independent variables for model inclusion. The best model was selected using Akaike's information criterion (AIC), a measure of the goodness of fit for statistical models (Furnival, 1971; Akaike, 1974; Neter & Wasserman, 1996), for this and all subsequent best subset regression. Regression coefficients based on standardized variables were calculated to compare the relationships between independent variables of differing magnitudes and the dependent variable.

We tested for geographic structure in AFLP marker data using analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992). This analysis was conducted in ARLEQUIN (version 2.000) based upon a genetic distance matrix and a hierarchical grouping matrix, with individual, plot and locality as grouping variables (Schneider *et al.*, 2000). To assess whether AFLP genetic markers were related to lesion size or phenotypic traits, we conducted a series of one-way ANOVAs, with presence or absence of a band as grouping factor and lesion area response or the phenotypic traits as dependent variables. Effect sizes (Hedges' $\hat{g}$) were calculated for each ANOVA (Hedges & Olkin, 1985) to allow for comparisons among dependent variables of different magnitudes. We used a best subset regression, as described above, to examine relationships between lesion area response and phenotypic traits, while accounting for loci significantly related to lesion area.

To examine the spatial structuring of symptomatic leaf count per tree, we used an ANOVA with plot and locality as random effects. We performed a linear regression between symptomatic leaf count per tree and lesion area to see if a direct relationship existed. As an asymptomatic tree in nature can result from many factors other than its susceptibility, the regression was run again excluding trees with a symptomatic leaf count of zero ($n = 17$). This analysis was further refined using an assignment test approach, where trees with the highest 10% symptomatic leaf counts were assigned a '1' and the remaining 90% of symptomatic trees were assigned a '2', and a logistic regression of symptomatic leaf count level with lesion area was run. To examine the relationship between field disease expression and phenotypic traits, we performed a best subset regression between cumulative symptomatic leaf count and water content, leaf length, width, area, toughness, DBH and relevant interaction terms. In addition, a best subset regression analysis was run comparing lesion area with symptomatic leaf count while also accounting for variation of molecular markers, phenotypic traits, and relevant interaction terms. To compare symptom levels with environmental variables measured at the plot level, we ran a best subset regression between mean plot symptomatic leaf count and elevation, longitude, latitude, TMI, mean annual precipitation, mean daily minimum, maximum and mean temperatures, SII, and relevant interaction terms. A final best subset regression analysis was run of symptomatic leaf count with the lesion area, molecular markers, phenotypic traits, environmental variables, and relevant interaction terms.

Results

Variation among individuals in susceptibility

Following incubation, symptoms caused by *P. ramorum* on *U. californica* (leaf tip necrosis and spotting, as described by Davidson *et al.*, 2002) were observed on 82.4% of inoculated *U. californica* leaves ($n = 1313$ of 1593). Mean lesion area per tree varied significantly among individual trees (Fig. 2; ANOVA: $F_{96,1138} = 1.9$, $P < 0.0001$). Of the 102 leaves tested as control leaves, 14 were contaminated with non-*P. ramorum* organisms and were not able to be scored for resulting lesion size. Of the remaining 88 leaves, 10 of 88 produced small

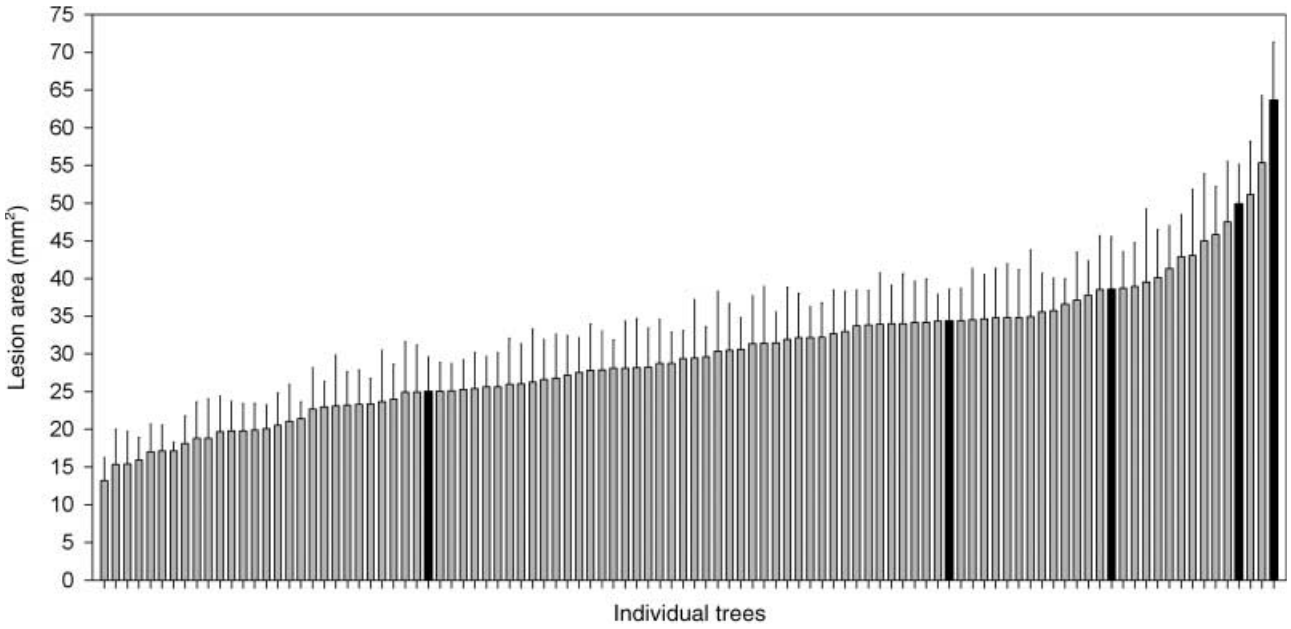

Fig. 2 Mean lesion area (±1 SE) per tree produced on detached leaves of *Umbellularia californica* collected from trees growing in Sonoma (gray bars, $n = 97$) and Marin (black bars, $n = 5$) Counties, California, USA, after inoculation with *Phytophthora ramorum* (isolate Pr52).

lesions (mean = 1.86 mm^2, SD = 1.28 mm^2, maximum = 4.0 mm^2). The leaves were plated to assess whether the pathogen could be recovered from the control leaves in culture. Of the 10 symptomatic control leaves plated for recovery of the pathogen, only one produced *P. ramorum* (1.2%). The pathogen was not recovered from any of the asymptomatic control leaves. The noncontrol leaves produced a significantly higher number of lesions (1313 of 1593). In addition, these leaves produced much larger lesions than controls (mean lesion size = 30.3 mm^2; SD = 60.8 mm^2; maximum = 1973.7 mm^2) and a much higher rate of recovery in culture was observed (67.3%). While the presence of small control lesions may suggest the presence of *P. ramorum* structures in asymptomatic leaves collected in the field, their occurrence likely makes a minor contribution to lesion areas produced following inoculation.

Mean lesion sizes following inoculation of *U. californica* with two different *P. ramorum* isolates (Pr52 and SSU2-1) were significantly positively related (standardized partial regression coefficient (m) = 0.49 ± 0.13 (± SD), $r^2 = 0.43$, $F_{1,20} = 15.1$, $P < 0.001$; Fig. 3). The local isolate (SSU2-1) consistently produced smaller lesions than the Pr52 isolate (paired t-test: $n = 22$, $t = 4.73$, $P < 0.001$), with the biggest differences observed on the most susceptible trees. Lesion area (resulting from infection by the Pr52 isolate) was positively related to the number of sporangia (ANCOVA: $F_{1,203} = 12.8$, $P < 0.001$), but the relationship between lesion area and sporangium number varied among individual trees (ANCOVA: $F_{26,203} = 1.6$, $P < 0.05$). Lesion area was also positively related to the presence of chlamydospores (Hedges' $\hat{g} = 21.7$, df = 1, $P < 0.0001$), and this relationship also varied among individual trees (Hedges' $\hat{g} = 54.8$, df = 26, $P < 0.0001$).

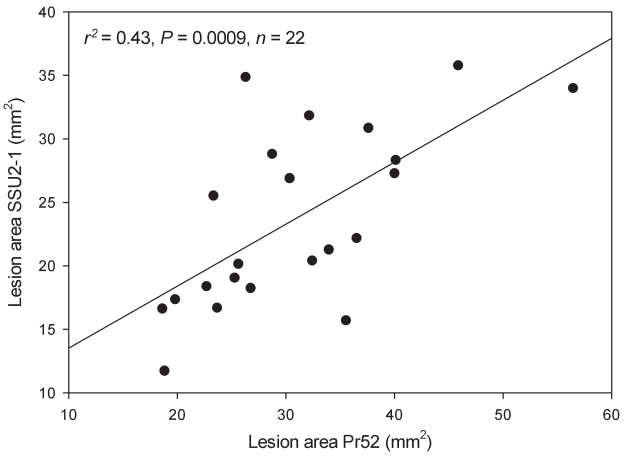

Fig. 3 Linear regression of mean lesion area per tree produced on detached leaves of *Umbellularia californica* after zoospore inoculation with two different isolates of *Phytophthora ramorum* (Pr52 and SSU2-1).

Variation in lesion size was not significant among plots or localities at $P < 0.05$ (Fig. 5a). However, the samples collected in China Camp State Park produced significantly larger lesions (ANOVA: $F_{1,100} = 10.5$, $P < 0.01$), but not significantly larger leaves, compared with all Sonoma plots ($P = 0.86$) (Fig. 5a).

Phenotypic and genotypic correlates of susceptibility

The results of the best subset regression analysis revealed significant relationships between lesion response to inoculation

Table 2 Best subset regression relating *Umbellularia californica* phenotypic traits and genotypic markers to mean lesion area produced per tree following inoculation with *Phytophthora ramorum* isolate Pr-52

Response and explanatory variables	r^2	df	*F*	*P*	Standardized regression coefficients
Phenotypic traits					
Mean lesion area per tree	0.40	3, 93	20.5	< 0.0001	
Mean leaf length per tree			16.7	< 0.0001	–0.66
Mean leaf area per tree			35.9	< 0.0001	+0.97
Mean leaf length × mean leaf area			11.7	< 0.001	–0.25
Phenotypic and genotypic variables					
Mean lesion area per tree	0.51	5, 91	19.1	< 0.0001	
Mean leaf area per tree			15.7	< 0.001	+0.33
bb146			26.3	< 0.001	+0.40
y121			6.1	< 0.05	–0.19
g187			13.3	< 0.001	–0.27
g398			9.3	< 0.01	+0.23

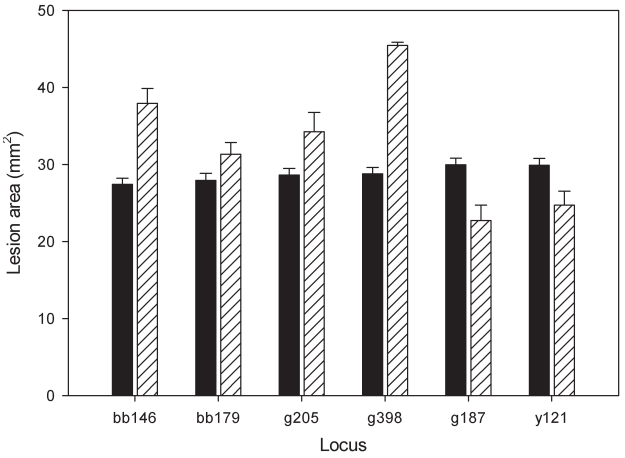

Fig. 4 Mean lesion area (±1 SE) and the presence (hatched bars) or absence (black bars) of amplified fragment length polymorphism (AFLP) bands at marker loci (each significantly related to lesion area at $P < 0.05$; see Table 3) in *Umbellularia californica* leaves.

and leaf length, leaf area, and their interaction (Table 2). No relationships were found between lesion area and water content, leaf toughness or DBH. Significant differences were observed in leaf area among plots (ANOVA: $F_{12,89} = 5.9$, $P < 0.001$). A model with leaf area and four of the six loci related to lesion area (see results in the next two paragraphs) explained 51% of the variation in lesion area (Table 2).

The three AFLP primer pairs used yielded 149 loci, 110 of which were polymorphic, and 100 of which produced banding patterns across samples that had correlation coefficients < 1.0 (thus not the same putative locus). The AMOVA test revealed significant spatial structuring of the molecular data, primarily at the tree-to-tree level, with 83.7% of variation within plots ($P < 0.0001$), 11.6% among plots within localities ($P < 0.0001$), and 4.7% among localities ($P < 0.0001$).

Our screening for individual loci related to disease response identified six loci significantly related to variation in lesion area (Table 3, Fig. 4). Loci associated with lesion area were present in every plot, although differences existed in their plot level frequency. Three of the six loci were also significantly related to phenotypic leaf traits (Table 3).

Factors influencing disease expression in nature

Phytophthora ramorum was recovered from 92% of the symptomatic *U. californica* leaves collected, confirming that field symptomatic leaf counts represented infection by the pathogen. In addition, no other foliar *Phytophthora* spp. were recovered. Symptomatic leaf count varied significantly among plots (ANOVA: $F_{11,85} = 18.1$, $P < 0.001$; Fig. 5b). Analysis at the plot level revealed that symptomatic leaf count is related to mean minimum annual temperature (°C), mean annual precipitation (mm), temperature moisture index (TMI), and longitude (Fig. 6). No relationships were found between symptomatic leaf count and tree phenotypic traits. Mean lesion area in our laboratory tests was not related to symptomatic leaf count directly ($r^2 = 0.004$, $P = 0.54$, $n = 97$). Also, relationships between symptomatic leaf count and lesion area were not found after asymptomatic trees had been excluded ($r^2 = 0.01$, $P = 0.31$, $n = 80$) or when assignment tests were used ($r^2 = 0.04$, $P = 0.13$, $n = 80$). Finally, multivariate analysis of the association between symptomatic leaf count and lesion area with the inclusion of phenotypic traits, molecular markers and environmental variables using a best subset regression approach did not result in significant relationships.

Discussion

The important role of *U. californica* in the incidence and spread of the invasive pathogen *P. ramorum* is well documented, but little is known about how and why *U. californica* trees differ

Table 3 One-way ANOVAs of *Umbellularia californica* phenotypic and disease traits (dependent variables) and presence/absence of amplified fragment length polymorphism (AFLP) loci (independent variables)

Locus	% of trees with locus	Leaf area	Leaf length	DBH	Lesion area	Symptomatic leaf count
bb179	36.1	0.32	0.26	0.23	0.42*	–0.01
g205	9.3	0.40**	0.77*	1.31**	0.69*	0.04
bb146	16.5	0.72**	0.34	0.06	1.44**	–0.20
g398	2.1	1.37	1.82*	–0.94	2.10**	–0.46
y121	14.4	0.24	0.55	–0.11	–0.64*	–0.61*
g187	11.3	–0.02	0.18	0.12	–0.91**	0.10

Effect sizes (Hedges' $\hat{g}$), significance values, and relationship direction are reported. Significance: $*P < 0.05$; $**P < 0.01$; df = 1, 95 for all ANOVAs. Loci present or absent in just one of the individuals surveyed were excluded from the analysis.
DBH, diameter at breast height.

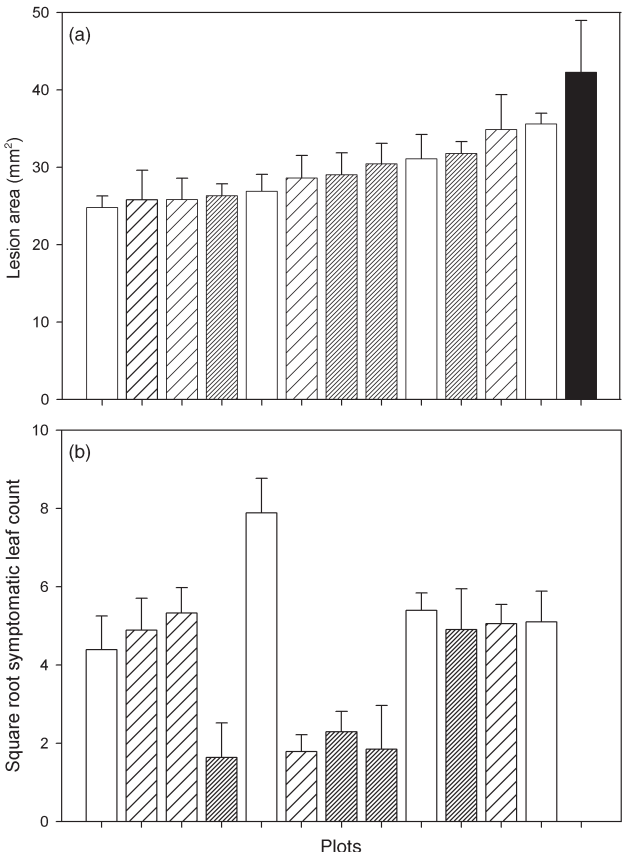

Fig. 5 Mean (±1 SE) per plot (a) lesion area and (b) square root symptomatic leaf count of *Umbellularia californica*. Fairfield Osborn Preserve (□); Sugarloaf Ridge State Park (▨); Annadel State Park (▨); China Camp State Park (■).

among individuals and populations in susceptibility to infection. This is the first study on the genetic and phenotypic correlates of susceptibility to *P. ramorum* in a key inoculum-producing host. We found six genetic loci that were significantly related to lesion size, three of which were also related to leaf phenotypic traits. Tree-to-tree variability in susceptibility was high, as was variation at the AFLP loci. Larger lesions were produced in the more southern Marin County locality than in the Sonoma County locality, suggesting that regional-scale differences in population-level susceptibility may exist. Also, we found that susceptibility of host leaves varied among *P. ramorum* isolates. However, laboratory-measured susceptibility of Sonoma trees was not related to disease expression in the field. Levels of disease expression in the field were most strongly related to abiotic conditions, suggesting that environmental setting is more important than susceptibility at the spatial scale of this study.

There was nearly a fivefold difference in foliar lesion area following inoculation of leaves with *P. ramorum*. No trees were found to be completely resistant, but each plot had a large range of susceptibility, which concurs with Hüberli *et al.* (2006). Lesion area was significantly related to the number of *P. ramorum* reproductive and resting spores, suggesting that more inoculum is produced from larger lesions, potentially creating a positive feedback between growing lesions and infection on neighboring leaves and trees. The finding that trees vary with respect to the relationship between lesion size and spore production illustrates how genetic variation among trees might influence their role as source for the spores that may eventually reach oak canker hosts. While we sampled from all plots on the same day to increase confidence that the differences among individuals and plots described were not confounded by the effects of seasonality, which affect both pathogen virulence and host response (Dowell, 2001; Meshriy *et al.*, 2006), phenological differences among trees may have influenced our lesion size results. Nevertheless, the fact that differences among genotypes in lesion size were similar from trees collected in different localities suggests that the genetic differences we observed represent general tree characteristics that affect the progress of infection on an individual host.

Lesion response per tree was positively correlated with mean leaf area and length, and the interaction of these two measurements. As we did not inoculate the whole leaf, but rather dipped a standard portion of the leaf tip (7–8 mm) into the *P. ramorum* solution, the resulting relationship between leaf and lesion size should not be a result of differing amounts of

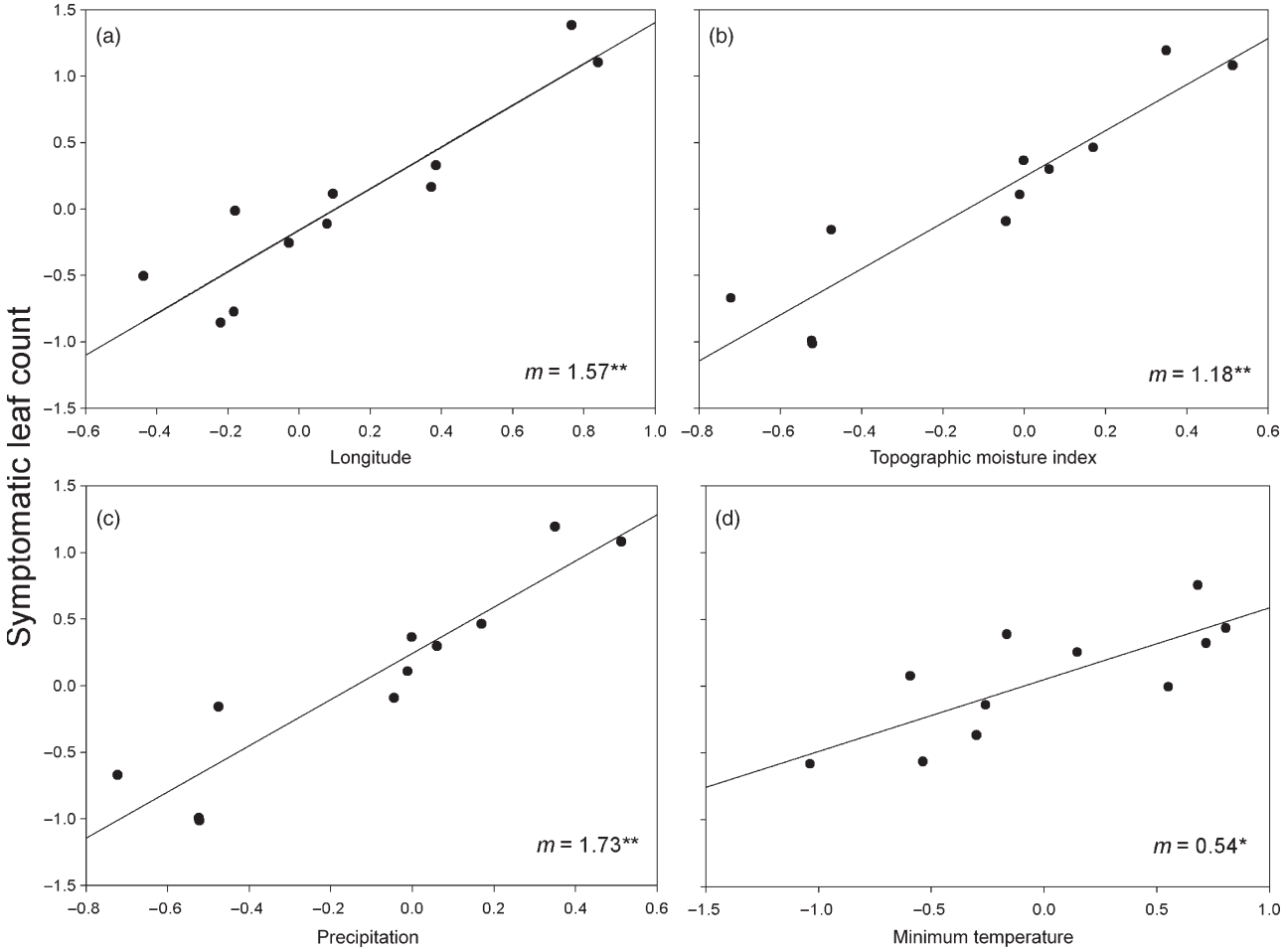

Fig. 6 Relationship of mean number of *Umbellularia californica* leaves with *Phytophthora ramorum* symptoms per plot with (a) longitude, (b) topographic moisture index, (c) mean precipitation, and (d) mean daily temperature minimum. *m*, standardized partial regression coefficient. Overall model fit $r^2 = 0.93$, $F_{4,6} = 19.7$, $P < 0.01$. Parameter significance symbols: $*P < 0.05$; $**P < 0.01$.

exposure to the pathogen during the inoculation trial. Rather, we believe that the relationship is more likely a result of structural or physiological changes occurring with leaf growth or leaf aging which facilitate infection by *P. ramorum*. Further work is needed to identify the nature of these leaf physiological factors.

Significant genetic structure was found at all spatial levels, but the majority of variation existed among individuals within plots. This is consistent with the pattern of variation in lesion response. The finding that genetic variation among plots was low supports anecdotal evidence that *U. californica* is an insect-pollinated, out-crossing species (Kasapligil, 1951). Of the 100 polymorphic loci scored, four were significantly related to larger lesions and two to smaller lesions. We interpret this to mean that only a few of the loci selected may confer traits related to disease resistance, which is not surprising given that the AFLPs are normally considered to be neutral markers selected randomly from across the genome (Lowe *et al.*, 2004).

With the markers we identified, we built a model that included four of the six loci as well as the phenotypic traits of leaf area that explained 51% of variability in lesion area. These results suggest a mechanism involved in determining foliar host susceptibility, where individuals with genes conferring larger leaves are more prone to infection. The residual variation may be accounted for by environmental factors not measured that affect expression of genotypic variation among trees.

Unlike lesion area, field disease level varied significantly among plots. We identified several environmental variables that were important to field disease level at the plot level, including mean minimum temperature, TMI, mean precipitation, and the spatial position variable longitude. This finding corroborates research showing that *P. ramorum* activity is dependent on environmental conditions, including temperature and humidity (Davidson *et al.*, 2005; Meentemeyer *et al.*, in press a). Disease expression in the field was not related to detached leaf susceptibility. Thus, we see that tree-to-tree genetic-based susceptibility at the landscape scale is overridden by the effects of local environmental conditions on pathogen activity. More thorough examinations of the relationship between forest microclimate and field-based disease severity are needed, with increased replication of sites and years.

Californian isolates of *P. ramorum* vary to a great extent in pathogenicity (Hüberli *et al.*, 2006). We found that foliar host susceptibility is significantly related across the two isolates compared on a subset of the leaf samples, indicating that the virulent isolate used for all samples interacts with *U. californica* similarly to the isolate found naturally in our plots. However, the virulent isolate produced significantly larger lesions, a result that is supported by previous findings. We are currently researching how variation in isolate virulence affects disease incidence and spread in the field.

Population variation in susceptibility to *P. ramorum* has been examined in other Californian hosts, namely the dead-end hosts *Quercus agrifolia* Nee (coast live oak, Fagaceae) and *L. densiflorus* (Dodd *et al.*, 2005; Hayden *et al.*, 2005). Lesion size in detached branch assays of *Q. agrifolia* individuals was found to vary within populations, with up to an eightfold difference, and not between populations. AFLP analysis supported this finding, with strong within-population and weaker among-population variation. Oaks are highly outbreeding species in which most of the genetic variance occurs within rather than between populations (Dodd & Kashani, 2003). Our study with a foliar host also found considerable differences in susceptibility within a locality. However, we cannot comment on the variation among populations across the endemic range, as samples were collected from a small localized region. Unlike the case of *Q. agrifolia*, we were able to correlate AFLP markers to susceptibility in *U. californica*.

In Californian forests, *P. ramorum* is an introduced pathogen of unknown origin (Garbelotto *et al.*, 2003; Ivors *et al.*, 2006). In the absence of coevolved resistance to an exotic pathogen, hosts are usually extremely susceptible, and often, but not always, the host range of the pathogen is broad, as for *P. ramorum*. However, resistance in forests trees to exotic *Phytophthora* spp. has been demonstrated previously, such as for *Phytophthora cinnamomi* on *Eucalyptus marginata* (jarrah, Myrtaceae) in Western Australia (Stukely & Crane, 1994) and *Phytophthora lateralis* on *Chamaecyparis lawsoniana* (Port Orford cedar, Cupressaceae) in Oregon (Oh *et al.*, 2006). In these examples, the data suggest that multigenic resistance is involved in the host–pathogen interaction which is also suggested for *P. ramorum* in our study with *U. californica* foliage.

In this study, we found that *U. californica* trees varied significantly in their ability to minimize infection by *P. ramorum* and we have identified several factors correlated with susceptibility and field disease expression. Measurement of phenotypic traits and the genetic composition of *U. californica* in conjunction with local environmental conditions enabled a more thorough understanding of the controls of inoculum build-up and spread in the sudden oak death pathosystem. Understanding the nature of susceptibility in the key inoculum-producing hosts will help us to better predict the risk of disease spread to oaks and impacts on the forest ecosystems of California and Oregon.

Acknowledgements

We thank Josh Amaris, Shelly Benson, Deanne DiPietro, Greg Garner, Andre Gauthier, Rich Hunter, Melina Kozanitas, Matthew Meshriy, Lori Miles, Steve Moyle, Amy Smith, Kirsten Ward and J. Djibo Zanzot for help with plot establishment, harvest and inoculation. Funding was provided by the National Science Foundation (DEB-0217064 and EF-0622677), the Betty and Gordon Moore Foundation, and the USDA Forest Service – Pacific Southwest Station.

References

Akaike H. 1974. A new look at the statistical identification model. *IEEE Transactions on Automatic Control* **19**: 716–723.

Alexander HM, Antonovics J, Kelly W. 1993. Genotypic variation in plant disease resistance-physiological resistance in relation to field disease transmission. *Journal of Ecology* **81**: 325–333.

Alexander HM, Thrall PH, Antonovics J, Jarosz AM, Oudemans PV. 1996. Population dynamics and genetics of plant disease: a case study of anther-smut disease. *Ecology* 77: 990–996.

Anderson, PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P. 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology and Evolution* **19**: 535–544.

Antonovics J, Thrall PH. 1994. The cost of resistance and the maintenance of genetic polymorphism in host-pathogen systems. *Proceedings of the Royal Society of London Series B – Biological Sciences* **257**: 105–110.

Beven KJ, Kirkby MJ. 1979. A physically based, variable contributing area model of basin hydrology. *Hydrological Sciences Bulletin* **24**: 43–69.

Burdon JJ, Jarosz AM, Kirby GC. 1989. Pattern and patchiness in plant–pathogen interactions-causes and consequences. *Annual Review of Ecology and Systematics* **20**: 119–136.

Carlsson-Graner U, Thrall PH. 2002. The spatial distribution of plant populations, disease dynamics and evolution of resistance. *Oikos* **97**: 97–110.

Condeso TE, Meentemeyer RK. 2007. The effects of landscape heterogeneity on the emerging forest disease sudden oak death. *Journal of Ecology* **95**: 364–375.

Daszak P, Cunningham AA, Hyatt AD. 2000. Emerging infectious diseases of wildlife–threats to biodiversity and human health. *Science* **287**: 443–449.

Davidson JM, Rizzo DM, Garbelotto M. 2002. *Phytophthora ramorum* and sudden oak death in California: II. Pathogen transmission and survival. In: Standiford R, McCreary D, eds. *Proceedings of the Fifth Symposium on California Oak Woodlands. USDA general technical report PSW-GTR-184*. Albany, CA, USA: USDA, 741–749.

Davidson JM, Wickland AC, Patterson HA, Falk KR, Rizzo DM. 2005. Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. *Phytopathology* **95**: 587–595.

Dodd RS, Hüberli D, Douhovnikoff V, Harnik TY, Afzal-Rafii Z, Garbelotto M. 2005. Is variation in susceptibility to *Phytophthora ramorum* correlated with population genetic structure in coast live oak (*Quercus agrifolia*)? *New Phytologist* **165**: 203–214.

Dodd RS, Kashani N. 2003. Molecular differentiation and diversity among the California red oaks section Lobatae. *Theoretical and Applied Genetics* **107**: 884–892.

Dowell SF. 2001. Seasonal variation in host susceptibility and cycles of certain infectious diseases. *Emerging Infectious Diseases* 7: 369–373.

Dubayah R. 1994. A solar radiation topoclimatology for the Rio Grande river basin. *Journal of Vegetation Science* **5**: 627–640.

Erwin DC, Ribeiro OK. 1996. *Phytophthora diseases worldwide*. St Paul, MN, USA: The American Phytopathological Society Press.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**: 479–491.

Frank SA. 1997. Spatial processes in host-parasite genetics. In: Hanski IA, Gilpin ME, eds. *Metapopulation biology: ecology, genetics, and evolution*. London, UK: Academic Press, 325–352.

Furnival GM. 1971. All possible regressions with less computation. *Technometrics* **13**: 403–408.

Garbelotto M, Davidson JM, Ivors K, Maloney PE, Hüberli D, Koike ST, Rizzo DM. 2003. Non-oak native plants are main hosts for sudden oak death pathogen in California. *California Agriculture* **57**: 18–23.

Gordon S. 2006. Landscape level genetic structure in *Umbellularia californica* (Hook. & Arn.) Nutt., a widespread forest tree species. Masters thesis. Sonoma State University, Rohnert Park, CA, USA.

Hayden K, Garbelotto M. 2005. Variation in susceptibility of tanoak to sudden oak death at the population and species levels. *Phytopathology* **95**: S41.

Hedges LV, Olkin I. 1985. *Statistical methods for meta-analysis*. San Diego, CA, USA: Academic Press.

Holdenrieder O, Pautasso M, Weisberg PJ, Lonsdale D. 2004. Tree diseases and landscape processes: the challenge of landscape pathology. *Trends in Ecology and Evolution* **19**: 446–452.

Hüberli D, Afzal-Rafii Z, Dodd RS, Douhovnikoff V, Harnik TY, Meshriy M, Miles L, Reuther K, Garbelotto M. 2006. Interactions of *Phytophthora ramorum* with two native Californian trees: bay laurel and coast live oak. In: Brasier C, Jung T, Oswald W, eds. *Progress in research on phytophthora diseases of forest trees. Proceedings of the Third International IUFRO Working Party S07.02.09 Meeting at Freising, Germany, 11–18 September 2004*. Farnham, UK: Forest Research, 79–83.

Hüberli D, Van Sant W, Tse JG, Garbelotto M. 2003. First report of foliar infection of starflower by *Phytophthora ramorum*. *Plant Disease* **87**: 599.

Ivors, K, Garbelotto M, Vries IDE, Ruyter-Spira C, Hekkert BT, Rosenzweig N, Bonants P. 2006. Microsatellite markers identify three lineages of *Phytophthora ramorum* in US nurseries, yet single lineages in US forest and European nursery populations. *Molecular Ecology* **15**: 1493–1505.

Kasapligil B. 1951. Morphological and ontogenetic studies of *Umbellularia californica* Nutt. and *Laurus nobilis* L. *University of California Publications in Botany* **25**: 115–239.

Kearsley MJC, Whitham TG. 1989. Developmental changes in resistance to herbivory: implications for individuals and populations. *Ecology* **70**: 422–434.

Kelly M, Meentemeyer RK. 2002. Landscape dynamics of the spread of Sudden Oak Death. *Photogrammetric Engineering and Remote Sensing* **68**: 1001–1009.

Lowe A, Harris SA, Ashton P. 2004. *Ecological genetics: design, analysis, and application*. Oxford, UK: Blackwell Publishing.

Meentemeyer RK, Anacker BL, Mark W, Rizzo DM. (in press b). Early detection of emerging forest disease using dispersal estimation and ecological niche modeling. *Ecological Applications.*

Meentemeyer RK, Rank NE, Anacker BL, Rizzo DM, Cushman JH. (in press a). Influence of land-cover change on the spread of an invasive forest pathogen. *Ecological Applications.*

Meentemeyer RK, Rizzo D, Mark W, Lotz E. 2004. Mapping the risk of establishment and spread of sudden oak death in California. *Forest Ecology and Management* **200**: 195–214.

Meshriy M, Hüberli D, Harnik T, Miles L, Reuther K, Garbelotto M. 2006. Variation in susceptibility of *Umbellularia californica* (bay laurel) to *Phytophthora ramorum*. In: Frankel SJ, Shea PJ, Haverty MI, eds. *Proceedings of the Second Sudden Oak Death Science Symposium, 18–21 January 2005. Monterey, California, USA*. USDA general technical report PSW-GTR-196. Albany, CA, USA: USDA, 131–134.

Moore ID, Grayson RB, Ladson AR. 1991. Digital terrain modeling: a review of hydrological, geomorphologic and biological applications. *Hydrological Processes* **5**: 3–30.

Neter J, Wasserman W. 1996. *Applied linear statistical models*. Homewood, IL, USA: Irwin.

Oh E, Hansen EM, Sniezko RA. 2006. Port-Orford-cedar resistant to *Phytophthora lateralis*. *Forest Pathology* **36**: 385–394.

Ostfeld, RS, Glass GE, Keesing F. 2005. Spatial epidemiology: an emerging (or re-emerging) discipline. *Trends in Ecology and Evolution* **20**: 328–336.

Rizzo DM, Garbelotto M. 2003. Sudden oak death: endangering California and Oregon forest ecosystems. *Frontiers in Ecology and the Environment* **1**: 197–204.

Rizzo DM, Garbelotto M, Davidson JM, Slaughter GW, Koike ST. 2002. *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. *Plant Disease* **86**: 205–214.

Rizzo DM, Garbelotto M, Hansen EM. 2005. *Phytophthora ramorum*: integrative research and management of an emerging pathogen in California and Oregon forests. *Annual Review of Phytopathology* **43**: 309–335.

Schneider S, Roessli D, Excoffier L. 2000. *Arlequin: A software for population genetics data analysis, version 2.000*. Geneva, Switzerland: University of Geneva.

Stukely MJC, Crane CE. 1994. Genetically based resistance of *Eucalyptus marginata* to *Phytophthora cinnamomi*. *Phytopathology* **84**: 650–656.

Swiecki, T. (in press). Distance from California bay reduces the risk and severity of *Phytophthora ramorum* canker in individual coast live oaks. In: Frankel SJ, Shea PJ, Haverty MI, eds. *Proceedings of the Third Sudden Oak Death Science Symposium, 5–9 March 2007*. USDA general technical report PSW-GTR. Albany, CA, USA.

Swiecki TJ, Bernhardt E. 2002. Evaluation of stem water potential and other tree and stand variables as risk factors for *Phytophthora ramorum* canker development in coast live oak. In: Standiford R, McCreary D, eds. *Proceedings of the Fifth Symposium on California Oak Woodlands*. USDA general technical report PSW-GTR-184. Albany, CA, USA: USDA, 787–798.

Thrall PH, Burdon JJ, Young A. 2001. Variation in resistance and virulence among demes of a plant host-pathogen metapopulation. *Journal of Ecology* **89**: 736–748.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. A new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

