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Research

Is variation in susceptibility to *Phytophthora ramorum* correlated with population genetic structure in coast live oak (*Quercus agrifolia*)?

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Summary

• California coastal woodlands are suffering severe disease and mortality as a result of infection from *Phytophthora ramorum*. *Quercus agrifolia* is one of the major woodland species at risk. This study investigated within- and among-population variation in host susceptibility to inoculation with *P. ramorum* and compared this with population genetic structure using molecular markers.

• Susceptibility was assessed using a branch-cutting inoculation test. Trees were selected from seven natural populations in California. Amplified fragment length polymorphism molecular markers were analysed for all trees used in the trials.

• Lesion sizes varied quantitatively among individuals within populations, with up to an eightfold difference. There was little support for population differences in susceptibility. Molecular structure also showed a strong within-population, and weaker among-population, pattern of variation.

• Our data suggest that susceptibility of *Q. agrifolia* to *P. ramorum* is variable and is under the control of several gene loci. This variation exists within populations, so that less susceptible local genotypes may provide the gene pool for regeneration of woodlands where mortality is high.

Key words: amplified fragment length polymorphism (AFLP), disease susceptibility, molecular marker, *Phytophthora ramorum*, population structure, *Quercus agrifolia*.

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Introduction

Recently, disease and mortality of a wide range of tree, shrub and herbaceous species native to the coastal woodlands of California and southern Oregon have been attributed to *Phytophthora ramorum*, an oomycete suspected to be of exotic origin (Rizzo *et al.*, 2002, Rizzo & Garbelotto, 2003). First noted in 1995, spread of this disease has been very rapid and is reaching epidemic levels in some populations of tanoak (*Lithocarpus densiflorus*), coast live oak (*Quercus agrifolia*), Shreve oak (*Quercus parvula* var. *shrevei*) and California black oak (*Quercus kelloggii*) along the central coast of California (Macpherson *et al.*, 2000). Detection of the same pathogen on members of the Ericaceae in Europe (Werres *et al.*, 2001) led to more detailed investigations of host species in coastal woodlands and forests of California (Rizzo *et al.*, 2002). Over 25 species from 15 families have now been identified as known hosts to *P. ramorum* (Davidson *et al.*, 2003; Rizzo *et al.*, 2003).

Current surveys suggest that there are two forms of host phenotypic responses (Davidson *et al.*, 2003). One is the formation of stem and branch cankers as found on members of the red oaks, section Lobatae of the genus *Quercus*, and on canyon live oak (*Quercus chrysolepis*) from section Protobalanus (Davidson *et al.*, 2003). The second is a leaf infection found in most of the other known host species. *Lithocarpus densiflorus* produces both types of phenotypic responses. Neither chlamydospores, nor sporangia have been recovered from stem cankers, which suggests that *P. ramorum* is unable to

complete its life cycle on oak hosts (Garbelotto *et al.*, 2003). However, sporulation from foliar infections appears to be an important source of inoculum (Davidson *et al.*, 2003). Preliminary studies suggest that proximity to foliar hosts may be an important cause of variation in incidence of infection in *Q. agrifolia* woodlands (Kelly & Meentemeyer, 2002; Swiecki & Bernhardt, 2002).

The four red oaks of California include one deciduous species (*Q. kelloggii*) and three evergreen species (*Q. agrifolia*, *Q. parvula* and *Quercus wislizeni*). Fine-scale habitat differentiation among them results in some differences in geographic ranges, but areas of sympatry are extensive, and all four species can be found in the coastal California woodlands. Until now, *P. ramorum* cankers have not been observed on *Q. wislizeni* in woodlands, although seedlings are susceptible to infection in the greenhouse (D. Rizzo, pers. comm.). It is unclear if *Q. wislizeni* is more resistant, or if it is an 'escapee' as this species occupies drier sites, or that it has been misidentified because of close morphological similarity to *Q. parvula* var. *shrevei*. Hybrids have been reported for all species combinations based on morphological, biochemical and molecular traits (Brophy & Parnell, 1974; Tucker, 1980; Vasey, 1980; Nason *et al.*, 1992; Dodd *et al.*, 1993; Dodd & A.-Rafii, 2004).

Mortality of *Q. agrifolia* from *P. ramorum* has been particularly severe along the central coast of California, in moderately humid closed forests and woodlands within the summer fog belt. *Quercus agrifolia* is restricted to coastal California and northern Baja California, Mexico. However, within this range it occupies a variety of habitats from sea level to altitudes over 1500 m in southern California and from humid coastal forest to savanna woodland at its eastern limits. It is likely that adaptive genetic variation exists among populations throughout this range of habitats. Geographic variations in biochemical traits that are likely to be correlated with environmental stress suggest that populations may form three groups: northern California, central California and southern California (Dodd & A.-Rafii, 1994; Dodd *et al.*, 1993, 1997). Except for biochemical variation, little is known of the genetic structure within and among populations of *Q. agrifolia*. Foliar morphology and tree architecture exhibit considerable variation, but with the exception of a southern form with heavily pubescent foliage (*Q. agrifolia* var. *oxyadenia*), morphological variation is at least as great within as between populations (R. S. Dodd, unpubl. data). *Quercus agrifolia* is a woodland species that has been seriously disturbed by urban development, establishment of vineyards and ranching activities that have resulted in many populations becoming small and fragmented. Understanding the capacity for gene flow among these populations will be important in predicting how this species will survive disease epidemics such as that resulting from infection by *P. ramorum*.

Although a gene-for-gene virulence-resistance mechanism is unlikely in this exotic pathogen–host interaction, differences in generalized resistance, or host physiological differences that indirectly interfere with disease progression may lead to variations in susceptibility. At a local woodland scale, infection is patchy, with considerable variation in levels of infection among and within sampling plots (Kelly & Macpherson, 2001). At this stage little is known of the mode of spread of this disease, however, this patchy pattern of infection is consistent with variations in tree susceptibility resulting from an uneven distribution of host genotypes. Variation in susceptibility of trees to artificial inoculation in the field adds further support to the potential for tree-to-tree differences in disease resistance (Kan-Rice, 2001).

The objectives of the research reported here were as follows. (1) To assess variation in susceptibility to *P. ramorum* among and within native populations of *Q. agrifolia* in California. Response to artificial inoculation of detached branch cuttings was used as an indication of potential differences in susceptibility of the host species. (2) To explore whether host molecular genotypes were correlated with variations in lesion length in response to inoculation among individuals and could therefore serve as predictors of susceptibility to *P. ramorum*. As molecular markers, we chose amplified fragment length polymorphisms (AFLPs), first described by Vos *et al.* (1995). This method combines the restriction fragment length polymorphism (RFLP) technique with the advantages of polymerase chain reaction (PCR) and detects dominant multilocus DNA fingerprints from selective PCR amplification. The great advantage of AFLPs over microsatellites is their applicability to DNA of any origin without prior sequence knowledge, primer synthesis and library construction. Their lower rate of mutation compared with microsatellites (Mariette *et al.*, 2001), also leads to greater population differentiation (Hedrick, 1999). Amplified fragment length polymorphisms have been used successfully to detect population differentiation in plants (Gaudeul *et al.*, 2000; Dodd *et al.*, 2002; Maguire *et al.*, 2002; Dodd & Kashani, 2003), in animals (Ogden & Thorpe, 2002; Creer *et al.*, 2004) and in microorganisms (Ivors *et al.*, 2004).

Materials and Methods

Sample locations

To capture as much potential genetic variation as possible within *Q. agrifolia*, seven populations were sampled including: (1) two populations in the northern range of the species, where the incidence of disease is high; (2) three populations along an east–west transect in central California; and (3) two populations from southern California. Population locations, codes and sample numbers are shown in Fig. 1 and Table 1. The populations from central and southern California were sampled twice during the season and individuals from the northern populations were included for each of the four sample dates.

Climate data for each population was estimated from the Daymet database developed by the University of Montana, Numerical Terradynamic Simulation Group and available at http://www.daymet.org/. Daymet generates daily surfaces

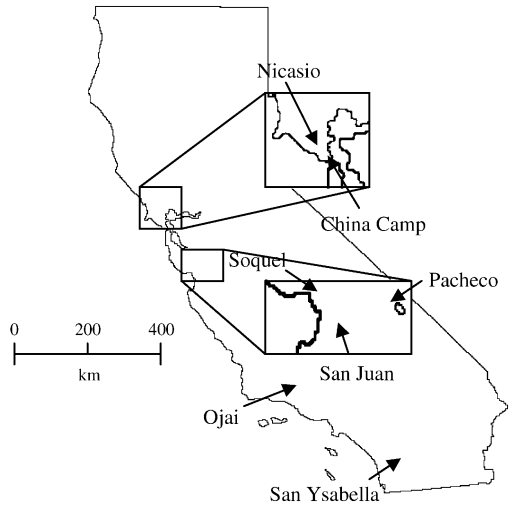

Fig. 1 Map of populations of *Quercus agrifolia* sampled for inoculation with *Phytophthora ramorum*.

Table 1 Details of population locations of *Quercus agrifolia* in California, dates collected, codes and sample size

Trial	Location	California	Sample size	Date collected
1	Nicasio	Northern	8	19 March 2002
	China Camp	Northern	11	
	San Ysabella	Southern	11	
	Ojai	Southern	11	
2	Nicasio	Northern	4	10 June 2002
	Pacheco	Central	10	
	Soquel	Central	9	
	San Juan	Central	10	
3	Nicasio	Northern	6	26 August 2002
	Pacheco	Central	10	
	Soquel	Central	9	
	San Juan	Central	10	
4	Nicasio	Northern	6	7 October 2002
	San Ysabella	Southern	10	
	Ojai	Southern	10	

See Fig. 1 for sample locations.

of temperature, precipitation, humidity, and radiation over complex terrain at a 1 km level of resolution, taking into account elevation. The model uses an 18-yr daily data set from 1980 to 1997. Monthly averages of temperatures (°C), precipitation (cm) and vapor pressure deficits (Pa) were obtained for each of the collection sites. A climate distance matrix was calculated using the Taxonomic distance metric in NTSYS-PC (Rohlf, 1993). A Mantel correlation was carried out between the climatic distance matrix and a Jaccards similarity matrix of AFLP molecular fingerprints.

Plant material

For each of the sample locations, about 10 adult trees were selected to have enough foliage for repeat sampling during the season. All trees were labeled. Branches were cut and their ends were wrapped in moist tissue during transport and then placed in buckets of water in a 12°C room, to avoid desiccation. Cuttings, approx. 1 cm in diameter at the base and about 25–40 cm long, were excised from the harvested branches. Requirements for selected cuttings were (1) long straight stems without lateral branches and (b) at least 8–10 healthy leaves. From each branch, five or six cuttings were selected to provide replication and controls for each individual tree. A sample of foliage of each individual was taken for DNA extraction and analysis. Branch cuttings were stood in jars of sterilized water covered with Parafilm (American National Can, Chicago, MI, USA), with the stems protruded through the Parafilm into the water.

Pathogen inoculum

Isolate Pr102 (ATCC MYA-2949) of *P. ramorum* was recovered from the stem canker of *Q. agrifolia* in Sonoma county, California, in 2001, and was used in all inoculations except Trial 1. In Trial 1, isolate Pr-52 (CBS 110537; ATCC MYC-2436) recovered from leaf lesions on a *Rhododendron* spp. in Santa Cruz County, California, in 2000, was used. A change in isolate was necessary because it was found after Trial 1 that Pr-52 was not from the main AFLP clone (Ivors *et al.*, 2004). Both isolates were found to be moderately to highly pathogenic on oak seedlings (D. Hüberli *et al.* unpubl. data). Inoculum discs, 5 mm diameter, were cut with a sterile cork borer from the margin of 14-d-old cultures grown at 20°C on vegetable-8 juice (Campbell Soup Company, Camden, NJ, USA) agar (Hüberli *et al.*, 1997) with omission of β-sitosterol and $CaCO_3$. The isolate is stored in the culture collection of D. Rizzo, University of California, Davis, CA, USA.

Inoculation and test conditions

A sterile cork borer was used to remove a 6 mm diameter disc of bark about 11 cm from the base of the branch cutting, well above its level of immersion in water. An inoculum disc was placed mycelium-side-down on the exposed cambial surface, the plug of bark was replaced and the inoculated area was wrapped with Parafilm and then silver Nashua tape (Tyco Adhesives, Norwood, MA, USA) for protection. For each individual tree, four or five replicate branch cuttings were inoculated. In addition, one branch cutting from each individual had the bark removed and replaced as described above, without the addition of inoculum. Branch cuttings were randomly placed on a greenhouse bench under natural day-lengths and ambient temperatures, with automatic misting. Supplemental water was given to the jars as needed. The average minimum and maximum temperatures for the four trials ranged from 15.4 to 16.1°C and 21.7 to 26.7°C, respectively.

An additional inoculation was conducted to investigate whether excision of branch cuttings from trees in the field altered their susceptibility. On 17 May 2002, attached branches

of four trees (NB, ND, NF and N4) from the Nicasio population were inoculated in the field and branch cuttings were inoculated in the greenhouse. Four small branches per tree, of the same diameter as the branch cuttings, were inoculated under the bark as described earlier.

Harvest and measurements

Branch cuttings were harvested after 21 d. Diameters of branch cuttings were measured at the site of inoculation. The outer bark around the site of inoculation was carefully scraped with a scalpel in order to expose the entire lesion. Vertical distances of infection above and below the site of inoculation and the maximum distance around the girth of the segment were recorded. Small pieces of tissue cut from the margins of the longitudinal lesion and the site of inoculation were plated onto pimaricin–ampicillin–rifampicin–pentachloronitrobenzene (PCNB) agar (P_{10}ARP) containing one-quarter the amount of PCNB, a selective medium for *Phytophthora* spp. (Erwin & Ribeiro, 1996), to confirm the presence of *P. ramorum*.

DNA sampling and analysis

Fresh leaves were washed in deionized water and their DNA extracted according to the Cullings modification of Doyle and Doyle (Cullings, 1992). DNA concentrations were assessed by electrophoresis on agarose gels and comparisons with lambda standards.

AFLPs

The AFLP method (Vos *et al.*, 1995) was performed using the AFLP Plant Mapping Kit following Applied Biosystems protocols (Applied Biosystems, Foster City, CA, USA). Approximately 100 ng of genomic DNA was used for digestion and ligation. Four *Mse*I/*Eco*RI primer pairs were used in the selective amplification step (*Mse*I-CAC/*Eco*RI-AC, *Mse*I-GTG/*Eco*RI-AC, *Mse*I-TC/*Eco*RI-AC and *Mse*I-TC/*Eco*RI-TC) These primers were chosen because they produced fingerprints with many well-defined polymorphic bands. The protocols for amplification were followed using a Techne Genius thermocycler (Techne Inc, Burlington, NJ, USA). Fingerprint data were obtained by running the amplified samples on an ABI PRISM 3100 genetic analyser (Applied Biosystems) followed by analysis with GENESCAN software. Band class selection was set for a fluorescence level five times greater than band detection for each sample and band detection used a ±0.5 bp range.

Data analysis

Analyses of variance were carried out on data from each of the trials separately and on combined data for the repeated trials (trials 1 and 4; trials 2 and 3) using the GLM procedure in SAS (SAS Institute Inc., Cary, NC, USA). Trials (T), populations (P) and trees (I) within populations were treated as random effects according to the following model:

$$Y_{ijk} = \mu + T_i + P_j + I(P)_{jk} + (TP)_{ij} + e_{ijk}$$

Where trials were treated separately, sets of linear contrasts were included to test the following hypotheses: (1) for trials 1 and 4, H_o (no difference between northern, China Camp and Nicasio, and southern, Ojai and San Ysabella, populations) and H_o (no difference between the two southern populations); and (2) for trials 2 and 3, H_o (no difference between northern, Nicasio, and central, Soquel, San Juan and Pacheco, populations) and H_o (no difference between coastal Soquel population and interior San Juan and Pacheco populations).

Association between genetic similarity of individuals and similarity in response to inoculation (lesion length) was tested under three hypotheses. (1) H_o: similarity in molecular profiles from AFLP fragments is uncorrelated with similarity in response to inoculation, measured by lesion size. Mantel tests were carried out between a pairwise distance matrix of lesion lengths and matrices of pair-wise similarities of AFLP banding patterns. A Euclidean distance was used as the metric for estimating lesion length distances. Genetic pairwise similarities were computed using Jaccards coefficient of similarity that only takes into account band sharing between individuals, lack of amplification of a band is not considered in the calculation of the similarity. Since population substructure may confound any of the associations between AFLP banding pattern and lesion sizes, partial Mantel tests were conducted in which a third matrix, representing membership of the same population, was partitioned out. This third matrix was a binary in which pairs of individuals from the same population were coded 1 and pairs of individuals from different populations were coded 0. Significance of all Mantel tests was estimated from 10 000 matrix permutations. (2) H_o: genetically similar individuals produce similar-sized lesions, whereas genetically dissimilar individuals may produce the full range of similarity in lesion sizes. This hypothesis assumes a nonlinearity in relationship between molecular similarity and lesion size similarity. To test this, binary matrices of five molecular similarity classes (99%, 95%, 90%, 75% and 50% most similar) were extracted from the original data. Pairwise molecular similarities were coded 1 if they were above the class limit and 0 if they were below the class limit. Simple and partial Mantel tests were carried out between the different molecular similarity classes and Euclidean distance matrices of lesion size and binary matrices of common population membership. (3) H_o: of the large number of anonymous AFLP markers, only a subset is correlated with lesion size in inoculated individuals. To test this, stepwise multiple regression analysis using the GLM procedure of SAS (SAS Institute Inc.) was carried out to determine the AFLP fragments that best explained variation in lesion size phenotypes. This was carried out separately for all

four trials. Of the fragments identified, 48 were common to at least two of the trials and were retained for further analysis. The Jaccard similarity matrix of the 48 AFLP molecular fragments was tested against matrices of three small lesion size classes. Lesions were coded 1 for small and 0 for large and the three size classes included the 10%, 25% and 50% smallest lesions, respectively. Mantel tests were carried out for the four trial dates separately.

Nei's (1978) unbiased genetic distances were estimated for all population comparisons using the pooled-primer AFLP data. Allele frequencies of dominant AFLP fragments were estimated using the method of Lynch and Milligan (1994), which is based on a Taylor expansion. A UPGMA dendrogram was determined using Tools for Population Genetics Analysis (TFPGA) (Miller, 1996) and support for nodes were estimated by bootstrapping with 1000 replications. Partition of molecular variance among and within populations was tested using WINAMOVA version 1.55 software (Excoffier *et al.*, 1992). Input files were prepared using AMOVA-PREP (Miller, 1998) using the simple matching coefficient which is a similarity measure based on the percentage of matches including both presence and absence of fragments. Levels of significance of covariance components in the analysis of molecular variance were estimated from distributions generated from 1000 random permutations.

Results

Branch cuttings survived well under the greenhouse conditions and by the end of each 3-wk incubation period, > 95% of the samples appeared healthy with some showing flushing of buds.

Response to inoculation

At 3 wk from inoculation, *P. ramorum* had caused visible lesions of varying dimensions, both vertically and horizontally. Control cuttings produced small lesions that, on average, were a little larger than the wound created. Although care was taken to standardize the diameter of branch cuttings, some variation was inevitable. Correlations of length of lesion with basal diameter of cuttings were nonsignificant at all sample dates (Table 2). However, the proportion of girdled branch circumference was significantly negatively correlated with its diameter. Smaller branch cuttings were more likely to be girdled than larger diameter branch cuttings, so we have not included this measure of response in further analyses.

The Shapiro-Wilks test showed no departure from normality of total lesion length for any of the four trials (trial 1 $W = 0.97$, $P = 0.48$; trial 2 $W = 0.98$, $P = 0.81$; trial 3 $W = 0.99$, $P = 0.96$; trial 4 $W = 0.99$, $P = 0.97$).

Variation in response to inoculation among populations

We were unable to include all populations in the same trial, but individuals from Nicasio in Marin were included in all four trials to provide a reference population. The results of nested analyses of variance for replicate trials of the northern and southern California populations and for the east–west transect in central California are shown in Table 3. Lesions produced in individuals from northern populations were significantly larger than in individuals from southern California for the combined trials and for trial 1 (Table 3). However, for trial 4, there were no significant differences among populations. Similarly, in trial 1, total lesion lengths were larger in individuals from San Ysabella than from Ojai, but no differences were found in trial 4.

Comparison of populations from the Central California transect with samples from Northern California failed to show significant differences in lesion size for either of the trials 2 and 3 (Table 3). No differences were detected in the comparison between the coastal population at Soquel and the more interior populations at San Juan and Pacheco. This lack of population differentiation in response to inoculation was also reflected in the analysis of the combined trials.

There appeared to be little evidence of differences in the symmetry in the lesion produced, as measured by length of lesion above and below the point of inoculation. Thus, we report only total lesion length here.

Variation in response to inoculation among individuals

In all four trials, significant differences were detected among individuals in lesion lengths. Plots of mean lesion lengths per individual showed a more or less linear increase (Fig. 2). To test for repeatability over successive trials, Spearman's rank order correlations were calculated for individual trees in trials 1 and 4 (northern and southern California) and in trials 2 and 3 (central California transect). In both cases correlations were

Table 2 Correlations between branch cutting diameter and size of lesions produced in *Quercus agrifolia* after inoculation with *Phytophthora ramorum*

Trial	Upper length	Lower length	Total length	Proportion girdled
1	−0.11 (0.16)	0.07 (0.37)	−0.02 (0.81)	−0.41 (< 0.0001)
2	0.06 (0.47)	−0.08 (0.30)	−0.01 (0.91)	−0.17 (0.03)
3	−0.03 (0.68)	0.03 (0.67)	−0.001 (0.99)	−0.48 (< 0.0001)
4	0.01 (0.90)	0.02 (0.83)	0.02 (0.84)	−0.52 (< 0.0001)

P-values are given in parenthesis. The upper and lower lesion lengths are vertical distances from the site of inoculation; proportion girdled is the proportion of circumference girdled.

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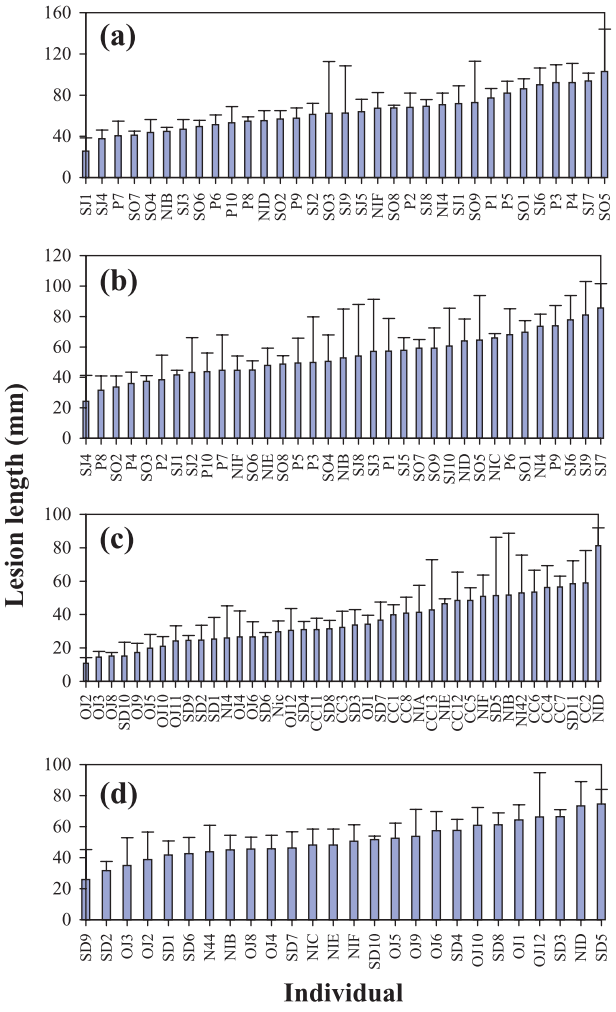
Table 3 Analysis of variance of total lesion length within and among populations of *Quercus agrifolia* in response to inoculation with *Phytophthora ramorum*

Trials 1 and 4 combined	df	*F*	*P*	Trials 2 and 3 combined	df	*F*	*P*
Inoculation date	1	56.8	< 0.0001	Inoculation date	1	12.0	0.0006
Population	3	8.4	< 0.0001	Population	4	0.5	0.71
Population × date	2	12.3	< 0.0001	Population × date	3	2.1	0.10
Individual (Pop)	37	2.6	< 0.0001	Individual (Pop)	31	3.5	< 0.0001
Individual × date	13	1.2	0.26	Individual × date	29	1.7	0.02
Error	229			Error	272		
Trial 1				Trial 2			
Populations	3	14.3	< 0.0001	Populations	3	0.4	0.78
North vs south	1	32.7	< 0.0001	North vs central	1	0.1	0.81
Ojai vs San Ysabella	1	6.2	0.014	Interior vs coastal	1	0.12	0.73
Individual (Pop)	35	1.7	0.02	Individual (Pop)	29	3.9	< 0.0001
Error	118			Error	134		
Trial 4				Trial 3			
Populations	2	0.3	0.72	Populations	3	2.8	0.04
North vs south	1	0.1	0.75	North vs south	1	2.2	0.14
Ojai vs San Ysabella	1	0.3	0.60	Interior vs coastal	1	0.1	0.74
Individual (Pop)	23	3.7	< 0.0001	Individual (Pop)	31	2.8	< 0.0001
Error	128			Error	141		



significant; for trials 1 and 4 (r_s = 0.26, P = 0.01) and for trials 2 and 3 (r_s = 0.19, P = 0.01). Plots of mean tree responses for the two trial dates for the two groups help to identify trees that consistently produced large lesions or small lesions (Fig. 3). For trials 1 and 4 (northern and southern California populations), individuals ND and SY5 consistently produced large lesions. For the same two trials, O2, O8, O3, SY10 and SY9 from the Ojai and San Ysabella populations consistently produced small lesions. As described earlier, there was no population structure in lesion size in trials 2 and 3, so individuals with consistently small or large lesions were independent of population location. Individuals SJ1, SJ4, P7, P8 and S4 produced consistently small lesions and SJ7, SJ6, P3, S1 and S5 produced consistently large lesions.

Seasonal variation in response

Analyses of variance indicated significant differences in lesion size between the two dates for the two sets of trials (Table 3). For trials 1 and 4 average lesion size increased from 28.7 mm (SE of mean s_Y = 2.4) on 17 March to 45.1 mm (s_Y = 2.1) on 7 October. Whereas for trials 2 and 3 average lesion size decreased from 61.29 mm (s_Y = 3.3) on 10 June to 51.3 mm

Fig. 2 Total lesion lengths produced on *Quercus agrifolia* branch cuttings after inoculation with *Phytophthora ramorum* at four trial dates. Repeat inoculations of an east–west transect on (a) 10 June 2002 and (b) 26 August 2002, and of northern and southern California populations on (c) 19 March 2002 and (d) 7 October 2002. Individual codes refer to populations: N, Nicasio; CC, China Camp; P, Pacheco; SJ, San Juan; S, Soquel; O, Ojai; SY, San Ysabella. Vertical bars represent one-sided 95% confidence interval for replicates of the same individual.

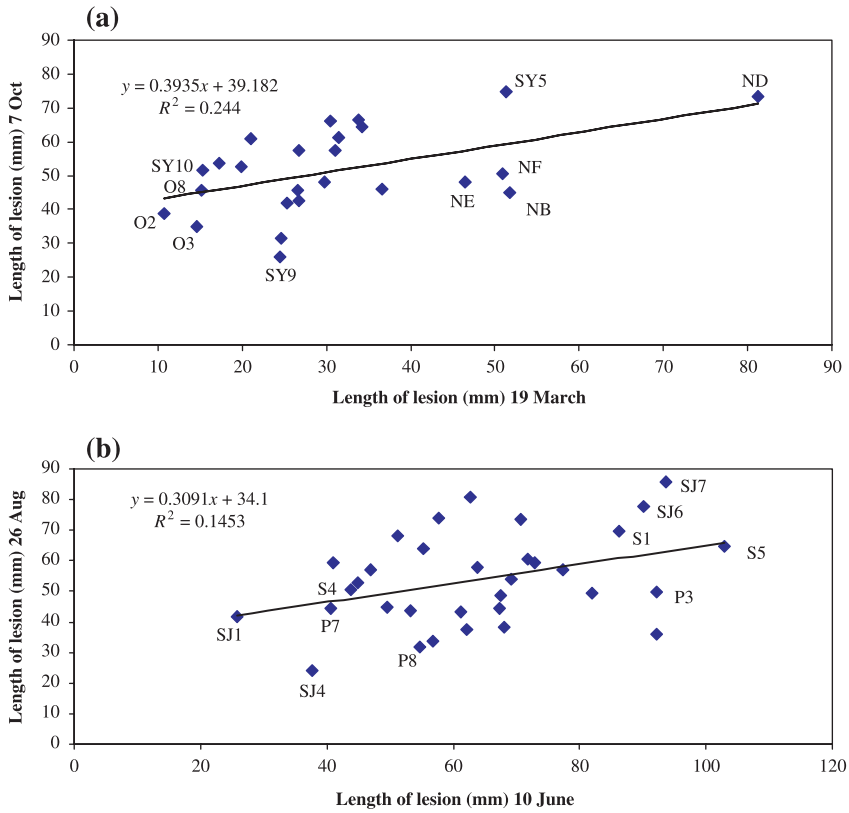


Fig. 3 Scatter plot of lengths of lesions produced on *Quercus agrifolia* branch cuttings after inoculation with *Phytophthora ramorum* at two trial dates. (a) Individuals from northern and southern California; (b) individuals from northern California and coast–interior transect in central California. Individuals with extreme responses are identified. Codes refer to populations: N, Nicasio; CC, China Camp; O, Ojai; SY, San Ysabella; P, Pacheco; SJ, San Juan; S, Soquel.

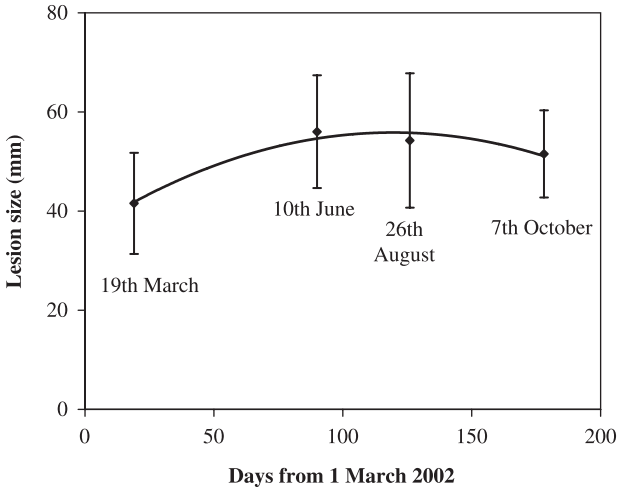


Fig. 4 Plot of mean lesion lengths produced on *Quercus agrifolia* branch cuttings after inoculation with *Phytophthora ramorum* at four trial dates. Trees are from Nicasio in northern California, USA. Vertical bars show 95% confidence limits. Fitted line is a quadratic polynomial.

(s_Y = 2.6) on 26 August An estimate of the sequence in lesion size over the four dates is shown in Fig. 4 for individuals of the Nicasio population that were included in all trials. This suggests a trend in which lesion size reached a maximum at the June inoculation and declined through the summer and early autumn.

Comparison of field inoculations and branch cutting inoculations

Only four trees were inoculated both in the field and under greenhouse conditions. Tree rankings for lesion size were identical under the two conditions and Pearson's correlation coefficient of lesion size under the two conditions was $r = 0.95$, $P = 0.05$. Of the four trees, N4 produced small lesions and D produced large lesions on attached branches in the field and on detached branch cuttings in the greenhouse.

Molecular correlates

The four AFLP selective primer amplifications produced 438 polymorphic fragments. Analysis of molecular variance indicated low, but significant population structure $\Phi_{ST} = 0.06$ ($P < 0.001$). A UPGMA cluster analysis based on the combined primer set revealed an interior clade including San Ysabella and Pacheco and a coastal clade including remaining populations (Fig. 5). Bootstraps provided good support for all nodes of the tree. A Mantel test revealed a significant correlation between a climatic distance matrix and a Jaccard matrix of molecular similarity ($R_M = -0.20$; $P = 0.04$).

Mantel tests revealed no significant correlations between similarities in overall AFLP molecular genotypes and pairwise distances in size of lesion produced in response to inoculation (Table 4). Negative Mantel correlations would be expected

Trial	Simple Mantel tests Lesion × genotype	Lesion × population	Partial Mantel tests Lesion × genotype.population
1	0.039	0.224***	0.011
2	−0.034	−0.065	−0.030
3	0.064	−0.048	0.079
4	−0.083	−0.030	−0.081

Common population membership removed in partial correlations.
***, $P < 0.001$.

Table 4 Mantel R correlation coefficients between similarity in amplified fragment length polymorphism molecular banding patterns of *Quercus agrifolia* and Euclidean distance in lesion size after inoculation with *Phytophthora ramorum* for each of four trials

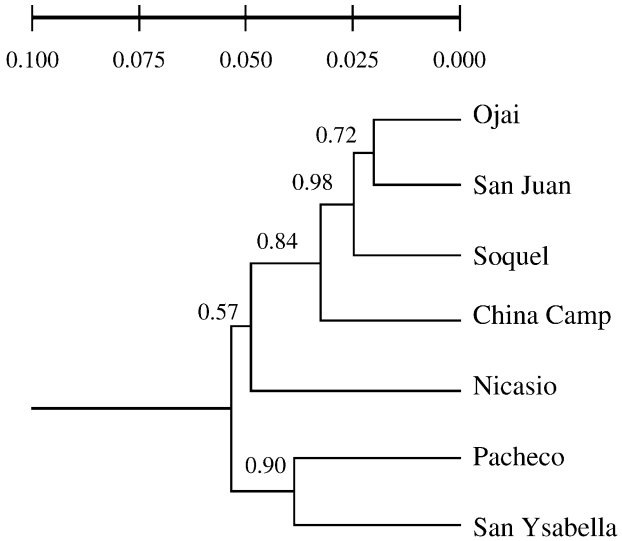


Fig. 5 UPGMA tree based on distances between populations estimated from Nei's unbiased genetic distance of amplified fragment length polymorphism (AFLP) fragments. Support for branches is given by bootstrap percentages for recalculated genetic distances from 1000 bootstrap replicates.

Molecular similarity	Trial 1	Trial 2	Trial 3	Trial 4
Up to 99%				
Lesion × genotype	0.022	0.040	−0.019	−0.04
Up to 95%				
Lesion × genotype	−0.05	0.03	−0.102*	0.05
Up to 90%				
Lesion × genotype	−0.05	−0.01	−0.053	0.070
Up to 75%				
Lesion × genotype	0.04	−0.04	0.05	−0.04
Up to 50%				
Lesion × genotype	0.01	0.02	0.02	0.10*

*, $P < 0.05$.

Table 5 Mantel R correlation coefficients between amplified fragment length polymorphism molecular similarity classes of *Quercus agrifolia* and Euclidean distances in lesion size of *Q. agrifolia* after inoculation with *Phytophthora ramorum* for each of four trials

between molecular similarity and Euclidean distance in lesion size. However, R_M values were not consistently negative. Common population membership was correlated with pairwise distances in lesion size only in the first trial, supporting the results from analyses of variance described earlier.

Selecting classes of the most similar molecular genotypes only marginally improved the association with distances in lesion size (Table 5). Significant correlations were found for Trial 3 (95% similarity class) and Trial 4 (50% similarity class). However, these correlations were not significant after the Bonferroni correction for multiple tests.

A reduced set of AFLP fragments was tested against matrices of small lesion size classes to investigate the possibility of fragments that were more closely correlated with the infection process. In this case, most simple Mantel tests returned significant correlations (Table 6). These correlations were highest in trials 1 and 4, which were replicated inoculation trials of the same individuals from northern and southern California. Mantel *R* correlations tended to be highest for the 25% small lesion size class. Partial Mantel tests removing effects of common population membership remained significant for the 25% small lesion size class for all trials.

Discussion

This is the first report of within-host species variation in susceptibility to *P. ramorum*. *Q. agrifolia* is a highly valued species in the coastal woodlands and urban areas of California and is suffering severe mortality from this pathogen. We have detected considerable variation in response to inoculation that is structured mostly at the level of individuals within populations, consistent with genetic structure in this oak species.

We had anticipated possible variation in susceptibility to *P. ramorum* associated with population adaptation to gradients of environmental stress. Xeric stress is likely to be the most important environmental constraint within the range of *Q. agrifolia* in California. Regional variation in resistance has been found for other exotic forest tree pathogens such as *Phytophthora cinnamomi* on *Eucalyptus marginata* (Stukely & Crane, 1994) and *Cronartium ribicola* on *Pinus lambertiana* (Kinloch, 1992), in which frequency of the major gene for resistance is relatively high in southern California populations. Whereas lesion size in *Q. agrifolia* varied significantly among individuals, there appeared to be little variation at the

Table 6 Mantel R correlation coefficients between similarity in selected amplified fragment length polymorphism (AFLP) molecular fragments and similarity in lesion size classes of *Quercus agrifolia* for each of four trials

Smallest lesion sizes	Trial 1	Trial 2	Trial 3	Trial 4
10%				
Lesion × genotype	0.119*	0.095	0.081	0.127*
Lesion × population	0.07	−0.042	0.073	0.07
Lesion × genotype.population	0.108*	0.096	0.061	0.121*
25%				
Lesion × genotype	0.150**	0.125*	0.151**	0.136*
Lesion × population	0.160**	−0.025	0.067	0.065
Lesion × genotype.population	0.115*	0.127*	0.151**	0.125*
50%				
Lesion × genotype	0.144**	0.074	0.101*	0.121*
Lesion × population	0.150**	−0.020	−0.008	−0.002
Lesion × genotype.population	0.125*	0.075	0.103*	0.122*

The 10% of AFLP fragments most closely associated with lesion sizes from canonical correlation analysis selected for analysis. Lesion size classes coded as binary similarity matrices with lesion sizes coded 1 for small and 0 for large lesions. Common population membership removed in partial correlations.
*, **, $P < 0.05$ and $P < 0.01$, respectively.

population level. Although southern California populations produced significantly smaller lesions than northern California populations in trial 1 (19 March 2002), this was not replicated in trial 4 (7 October 2002). No differences were detected in lesion size among populations from the central California transect and populations from northern California in either Trial 2 (10 June 2002), or Trial 3 (26 August 2002). The coastal populations from northern California occupy the most mesic conditions of the populations screened in our tests. These occupy the summer fog belt and are at locations where disease and mortality have been the most severe. The interior central California population from Pacheco and the southern California population from San Ysabella are the most xerically stressed of those that we sampled in this study. The differences in lesion size observed in Trial 1 are consistent with adaptation to xeric stress and the nonreproducibility in trial 4 could result from a seasonal change in physiology of the host. Variations in host response among samples from Nicasio that were replicated over the four trial dates, suggested a possible seasonal trend. We are currently investigating this with more intensive seasonal sampling. In inoculations of *Q. rubra* with *P. cinnamomi* at different times of the year, tree-to-tree variation in susceptibility could not be accounted for by phenological differences in the host alone, but appeared to depend upon some other intrinsic, or biochemical factor (Robin *et al.*, 1994). An alternative explanation for the lack of replication of the population effect in trials 1 and 4 could be the different strain of *Phytophthora* used in these two trials. Previous inoculation tests have shown that Pr-52, used in trial 1, is a highly pathogenic strain and Pr102, used in trial 4, is of intermediate pathogenicity (D. Huberli, unpubl. data). This should become clearer with the seasonal sampling that we are currently conducting.

Variation in lesion size among individuals within populations was significant for all trials, suggesting important tree-to-tree differences in susceptibility. This greater within- than among-population variance in lesion size was consistent with our estimates of the partition of molecular variance. Only 6% of AFLP molecular variance was attributed to populations. Oaks are highly outbreeding species in which most of the genetic variance occurs within, rather than between populations (Dodd & Kashani, 2003). Phenotypic response to inoculation appears to be partitioned in a similar way as molecular genetic variance. In all four trials, lesion sizes among individuals were normally distributed, suggesting a quantitative trait under the control of multiple gene loci. The range in variation was associated with date of inoculation and not with the populations screened. This was eightfold in trial 1, fivefold in trial 2 and threefold in trials 3 and 4, further suggesting a seasonal variation in either the pathogen, or in the physiology of the host. Lesion sizes were significantly correlated between the repeat trials. Some individuals could be identified as consistently producing small or large lesions, lending further support for tree-to-tree variation in susceptibility that might help explain some of the patchiness in mortality that has been observed (Kelly & Macpherson, 2001). Since *P. ramorum* varies in its pathogenicity to *Q. agrifolia* (D. Hüberli *et al.*, unpubl. data), more isolates need to be assessed to confirm if individual trees identified as less susceptible are indeed less susceptible across all isolates. In the *E. marginata* resistance screening program, a few individuals identified as resistant to one isolate of *P. cinnamomi* were subsequently found to be susceptible when inoculated with many isolates and under a range of different conditions (Stukely & Crane, 1994; Hüberli *et al.*, 2002a, 2002b).

Total AFLP molecular structure was not a good predictor of lesion size. We reduced the data set to include only genetically similar individuals, assuming that these might produce lesions of similar size. This improved correlations between AFLP molecular structure and lesion size slightly, but still did

not provide strong support for being able to use this molecular structure as an indicator of response to inoculation. The number of fragments detected by the AFLP procedure can be high and it is presumed that these fragments are drawn from across the entire genome. Relatively few, if any, of the fragments may be linked to regions that play a role in disease resistance. It is interesting that the dendrogram based on AFLP fragments produced a well-supported partition into an interior clade from Pacheco and San Ysabella and a coastal clade including remaining populations. This suggests that the molecular structure may have resulted from population origins from different geographic regions, perhaps following glacial refugia, or that an important proportion of the fragments are associated with environmental adaptation. The significant Mantel correlation between climate and AFLP fingerprints provides some support for this latter hypothesis. Thus, many of the AFLP fragments may be informative for population genetic history and for selection of adaptive traits. More intensive screening of these anonymous markers is necessary for detection of fragments associated with disease resistance genes.

Stepwise multiple regression allowed us to reduce the number of fragments to 48, approx. 11% of the total polymorphic fragments detected. Each of these was identified in the regressions for at least two of the trials. As would be expected, molecular structure based on these fragments was correlated with lesion size, particularly when the 25% smallest lesions were treated as a class. It may be assumed that individuals producing small lesions carry common genes that confer lowered susceptibility, whereas individuals producing large lesions may exhibit a broad range of genetic similarity. At this stage we are unable to say whether common genes are by descent, and hence imply relatedness, or whether they are common only by state, with no implied relatedness. We are currently carrying out progeny trials that should help resolve this.

Our results indicate that the branch-cutting assay method is a reliable technique for the detection of variation in the response to inoculation of *Q. agrifolia* with *P. ramorum*. Lesions produced *in vitro* were correlated with those produced *in vivo*. In addition, individuals were identified that produced consistently small and large lesions in the two inoculation trials. An *in vitro* method for screening host genotypes under controlled conditions is a necessary prerequisite in the detection of differential susceptibility to this pathogen by allowing large-scale and rapid screenings of individuals with minimal impact on trees in the field.

Although the exact mode of entry of the pathogen in *Q. agrifolia* is not well understood, infection occurs through the bark of major stems. In other *Phytophthora* spp., such as *P. cinnamomi*, zoospores have been shown to penetrate suberized stem tissue without conspicuous wounds (O'Gara *et al.*, 1996). Our underbark inoculation method bypasses the infection process proper; rather, it simulates pathogen colonization from a site in the cambial layer loaded with large amounts of inoculum. Although this method is biased in favor of pathogen colonization, it offers the advantage of eliminating uncontrollable variation in bark fissures, a likely infection court for the pathogen. This approach does not account for possible host–pathogen interactions that may take place on the surface or in superficial host tissues, and may add a further element of host resistance to *P. ramorum*. The bias in favor of the pathogen and the exclusion of further resistance mechanisms on the plant surface is a very conservative assay that makes the finding of reduced susceptibility in some individuals very significant.

Phytophthora ramorum is believed to be an exotic pathogen in the coastal woodlands of California (Garbelotto *et al.*, 2003). In the absence of evolution of a host–parasite system between this pathogen and *Q. agrifolia*, a gene-for-gene regulation of virulence and resistance is unlikely. However, there are examples of both simple and complex resistance of exotic forest pathogens. Exotic pathogens that infect tissue other than leaves usually involve complex resistance. For example, the data for the interaction between *P. cinnamomi* and *E. marginata* suggests multiple gene resistance (Stukely & Crane, 1994). By contrast, white pines exhibit single gene resistance against *C. ribicola*, white pine blister rust. If single gene resistance exists in coast live oak it is likely to be at very low frequencies.

Conclusions

There appears to be significant variation among trees of *Q. agrifolia* in response to inoculation with *P. ramorum* that may help explain some of the patchiness of disease and mortality that has been observed and indicates that there is potential for selection in natural populations. Trees with reduced susceptibility were present in all sites investigated, and are the most likely candidates to survive the predicted cyclic epidemic waves of sudden oak death and provide seed for the regeneration of *Q. agrifolia* populations that have been severely decimated by this disease. If reduced susceptibility is genetically inherited in significant proportions among offspring, seed may be used to produce less susceptible planting stocks for reforestation programs (assuming no adverse genetically correlated traits).

Sudden oak death is an emergent disease caused by a putative exotic pathogen. This is the first report documenting: (1) the presence of variations in susceptibility in the most popular host affected by this disease, and (2) the population structure of this trait in relation to the geography of California and to the molecular genetic architecture of oak populations, Finally, this study suggests that the variation in susceptibility may be controlled by multiple genes. Although single gene resistance has been reported for noncoevolved host–pathogen interactions, the presence of a multigene model is not surprising. Further research is needed to determine whether susceptibility to *P. ramorum* may be linked to specific phenotypic traits. This knowledge would be extremely useful for identification of less susceptible genotypes to be used in restoration programs. It

will also be interesting to determine whether the presence of *P. ramorum* is exerting a selection pressure strong enough to selectively shift the mean susceptibility level of populations exposed to the pathogen.

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