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Variation and heritability of host susceptibility to the introduced pathogen *Phytophthora ramorum* in tanoak (*Lithocarpus densiflorus*, proposed name *Notholithocarpus densiflorus*) populations

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Publication Date

2010

Peer reviewed|Thesis/dissertation

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By

Katherine J. Hayden

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Environmental Science, Policy, and Management

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Matteo Garboletto, Chair
Professor Richard Dodd
Professor Ellen Simms

Spring 2010

Abstract

Variation and Heritability of Host Susceptibility to the Introduced Pathogen *Phytophthora ramorum* in Tanoak (*Lithocarpus densiflorus*, proposed name *Notholithocarpus densiflorus*) Populations

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This dissertation presents a body of work collected from the very beginning of the outbreak of the introduced forest pathogen *Phytophthora ramorum*. The first data were taken in 2002, the year that *P. ramorum* was identified as the causal pathogen of the disease in California. The research, aimed at gathering the scientific knowledge necessary for an understanding and informed response to a new problem, placed an emphasis on bridging evolutionary ecological theory and management practice. First, my colleagues and I outline two quantitative polymerase chain reaction (qPCR) diagnostic assays for *P. ramorum* and their use as part of a widespread survey, screening California plants for the pathogen. In Chapter 3, my colleagues and I report the first surveys of variation in susceptibility to *P. ramorum* on detached leaves of field-collected tanoak trees. We found low but non-zero heritability of susceptibility, and that the structure of phenotypic variation in this measure of susceptibility echoed that of the neutral markers. Finally, I describe the establishment of a greenhouse common garden population of tanoak seedlings concurrently with a field test garden in an infested natural area. Using these study populations, we calculated the heritability of susceptibility in detached leaves with much greater precision and expanded the assay techniques and types of host resistance we measured. In so doing we

discovered highly variable responses of seedling families to intact-seedling tip inoculations, the majority of which variation was due to genetic factors. Using the qPCR detection techniques described in Chapter 2, we confirmed seedlings' infection within the first 7 months of the field test garden's establishment, with disease incidence rising over the course of the study. We found that both *P. ramorum* infection status and family origin have significant effects on tanoak survival in natural disease conditions. Together with the laboratory assays, our findings show that tanoak does possess some forms of genetic resistance to *P. ramorum*. The gardens we established provide the infrastructure necessary to determine if this resistance affects survival under natural conditions, to further investigate its inheritance and action, and to test the effect of varying environment and host genotype on its expression.

To Owen and Ada

INTRODUCTION

The emergent global trade of plants and wood products has brought with it a global movement of plant pathogens and pests—as well as disastrous consequences for forest ecosystems (Brasier 2008, Perrings 2002). Over the past 200 years, introduced pathogens have caused disease epidemics with far-reaching effects in ecosystems across the world. Chestnut blight (*Cryphonectria parasitica*) is a prime example of such consequences. First introduced to the United States around 1900 on Japanese chestnuts (Milgroom et al. 1996), within 50 years it reduced the American chestnut (*Castenea dentata*) from keystone species to understory shrub, altering fundamental ecosystem and causing a collapse of the economy that was centered around it (Anagnostakis 1987, Ellison 2005).

Before *C. parasitica* made its appearance, however, American chestnuts were already suffering from another exotic introduction. Chestnut ink disease, a root rot caused by the generalist pathogen *Phytophthora cinnamomi* which had been first introduced in the mid-1800s, (Anagnostakis 1995, Crandall et al. 1945). The same pathogen is responsible for jarrah dieback in western Australia, one of the examples of extreme mortality slinked to the introduction of an exotic pathogen (Hardham 2005). *Phytophthora cinnamomi* causes plant diseases in both agricultural and forest systems worldwide with varying degrees of virulence, but as the cause of Jarrah dieback it has been reported to kill 50-75% of the species in sites in Western Australia, in some cases leaving every tree and much of the understory dead (Weste 2003).

The conservation of species and natural ecosystems in the face of emergent diseases calls for the marriage of evolutionary ecological theory with management practice. The study of newly emergent diseases has the benefit of at least a century's worth of data from past epidemics (reviewes for example by Anagnostakis 1987, Kinloch 1993), as well as an increasingly well-

developed body of theoretical studies with which to predict disease dynamics and the potential for host-pathogen coevolution (Parker & Gilbert 2004). Accurate predictions and successful management strategies must account for these dynamics.

Biological introductions, and pathogen introductions in particular, hold the potential to drive rapid evolutionary change (Lambrinos 2004, Parker & Gilbert 2004, Suarez & Tsutsui 2008). While introduced pathogens are by no means guaranteed to be especially virulent (Desprez-Loustau et al. 2007, Linzer et al. 2009), mass host mortality seen in epidemics like the single-host chestnut blight or the landscape-wide jarrah dieback seems to be a hallmark of recent introductions (Burdon 1987, Parker & Gilbert 2004), or perhaps the inevitable result of a pathogen making an occasional sudden jump to a newly available, naïve host population (André & Day 2005). Even mortality that is randomly distributed across hosts, independent of their individual genotypes, can cause a reduction in host genetic diversity if it is in sufficient numbers (Lowe et al. 2007). On the other hand, the very introduction of pathogen to a new environment and host population may favor its rapid evolution to allow it to survive and exploit the newly available resource (André & Day 2005, Lee 2002, Reznick & Ghalambor 2001). Additionally, novel host-pathogen interactions present the potential for coevolutionary dynamics.

While classic coevolutionary models of animal systems suggest that, with exceptions depending on life histories and transmission dynamics, pathogens should evolve towards intermediate virulence over time to maximize the opportunities for transmission (Anderson & May 1982, Bull 1994, Frank 1996), the applicability of these models to plant systems has been called into question (Parker & Gilbert 2004). In particular, the associations between species described by the animal models are decoupled when the host may be infected with more than one pathogen genotype, when the pathogen can be transmitted by multiple hosts, or when the

pathogen can live freely outside the host for some part of its lifecycle, as do the resting spore stages of many fungi and *Phytophthoras* (Bergelson et al 2009, Jarosz & Davelos 1994, Woolhouse et al. 2001, Caraco & Wang 2008). Jarosz & Davelos (1994) review evidence that even the specialist *C. parasitica* has shown no evidence of favoring reduced virulence, despite the near-extinction of its host. Van Valen (1973), the originator of the Red Queen hypothesis that has morphed into the classic model of plant-pathogen coevolution, posited interspecific interactions primarily as the drivers of extinctions, rather than coexistence: "no species can ever win, and new adversaries grinningly replace the losers." The evidence Jarosz & Davelos (1994) compiled would seem to support Van Valen's dark view.

Recent models specifically surrounding plant-pathogen interactions allow for a patchy, interconnected distribution of host and pathogen (e.g., Barrett et al. 2008, Kirchner & Roy 1999, Thompson & Burdon 1992, Thrall & Burdon 1997). These models point to interactions of life history, spatial structure, and gene flow as determining the potential for hosts and pathogens to adapt with respect to the other, and predict variable, fluctuating outcomes. For example, where a pathogen is distributed unevenly across a host range, the potential for host adaptation in an infested zone may be swamped by the flow of susceptible genotypes through immigration or gene flow from a nearby disease-free area; conversely, a declining host population may be rescued by immigration and allowed to persist long enough to allow adaptation to occur (Garant et al. 2007, Nuismer et al. 1999)

Coevolutionary dynamics may also be affected by mechanisms of host resistance. The iconic plant pathogen resistance mechanism is known as R-gene resistance, whereby a pathogen protein (coded for by the avirulence gene, generically called Avr) is recognized by a plant receptor (coded for by the resistance, or R, gene), instituting a hypersensitive response, in which

programmed cell death walls off the infection (Flor 1956). In the gene-for-gene model of coevolution, the pathogen can gain virulence through evolution by losing or modifying the effector and so evade detection; this loss can be overcome by the gain of a new R-gene by the host, and so on (note that in the context of gene-for-gene coevolution, virulence means specifically the ability to infect, independent of the extent of infection). This model has much molecular support: for example, Jiang and colleagues (2008) found that a single, rapidly evolving family of genes has more than 370 members in each of two different *Phytophthora* species, and Hall and colleagues (2009) found that an extremely diverse R-gene in *Arabidopsis thaliana* is accompanied by extreme allelic diversity in the matching pathogen effector protein, supporting the gene-for-gene view of interaction. R-gene resistance is usually thought of as being qualitatively expressed and coded for by a single gene; however, exceptions to both exist (Bent & Mackey 2007). Resistance types that are underlain by one or few genes are frequently referred to as major gene resistance, whether or not it is related to an R-gene.

All other types of resistance are often lumped into a broad category of "quantitative resistance." These may include plant basal immunity, wherein pathogen proteins or molecules that are not specific to pathogenesis are recognized by the host and trigger an immune response (reviewed by Bent & Mackey2007), or phenological or morphological traits that in some way limit infection extent or incidence (Carson & Carson 1989, Sniezko 2006). Quantitative resistance may be underlain by one, few, or many genes; regardless, plant basal immunity and morphological traits are believed to be more difficult than R-gene resistance to break down or erode by rapid pathogen evolution (McDonald & Linde 2002, Carson & Carson 1989, but see Bishop 2000)

Relative to microbial pathogens, forest trees have a distinct disadvantage in generation time. While most pathogens complete their lifcycle in less than a year, a fast-growing tree can take 10 years or more to reach reproductive age. This one-sidedness makes rapid coevolution unlikely (Herrera 1983). While it is doubtless possible for forest trees to evolve resistance to novel pathogens – all species interactions must have been new at one point, after all – instances of evolutionarily aquired increased resistance will certainly be interspersed with failures and host extirpation. For species of special ecological or economic concern, active management should be considered to foster a response to a new, virulent pathogen. Resistance breeding programs have long been in place for a number of highly valued trees, for example, programs for western white pine (*Pinus monticola*) and sugar pine (*P. lambertiana*) have repeatedly selected and bred trees for resistance traits found in native populations for resistance to *Cronartium ribicola*, which causes a virulent rust on all five-needle pines. Both major-gene resistance and a number of forms of quantitative resistance were selected for; over a 30 year trial, major gene resistance broke down with a shift in pathogen population, while seedlings with some forms of quantitative resistance had the highest survival (Kinloch 2008).

Phytophthora ramorum is among the latest emergences of a novel, virulent forest pathogen that threatens extirpation of a key host. It was first noticed almost simultaneously in European nurseries and in dying oaks (*Quercus* spp.) and tanoaks (*Lithocarpus densiflorus* Hook. & Arn., proposed name *Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S. Oh, comb. nov) in the exurban-wildland interface in Marin County, California in the mid-1990s (Rizzo et al. 2002, Werres et al. 2001). Its origins have been traced to infected nursery stock of ornamental rhododendrons, with separate introductions to the United States and Europe, and more than one U.S. introduction (Ivors et al. 2004, Mascheretti et al. 2008). *P. ramorum*

causes a suite of diseases encompassed by the name ramorum blight and dieback: (i) a canker disease, also known as sudden oak death, which causes fatal trunk cankers on oaks and tanoaks; (ii) a twig blight that causes foliar symptoms and twig dieback on tanoaks, ericaceous, and a number of other hosts; and (iii) a leaf blight, characterized by solely foliar symptoms, most notably on California bay laurel/Oregon myrtlewood (*Umbellularia californica*) (Davidson et al. 2003).

While in Europe the disease has remained primarily in nurseries and gardens (Brasier 2008), from the very beginning it was clearly a threat to California forests. Oaks and tanoaks are a foundational species in oak woodlands, while in mixed evergreen forests tanoaks are the both primary masting species, supporting wildlife (Peterson 2002) and are the primary ectomycorrhizal host, supporting below-ground communities and nutrient cycling (Bergemann & Garbelotto 2006).

This dissertation presents a body of work collected from the very beginning of the outbreak; the first data were taken in 2002, the year that *P. ramorum* was identified as the causal pathogen of the disease in California (Rizzo et al. 2002). Therefore, the research questions move from the very general to the more specific, aimed at gathering the scientific knowledge necessary for an understanding and informed response to a new problem. Throughout, a focus was placed on bridging evolutionary ecological theory and management practice.

First, my colleagues and I asked among the most basic but necessary questions in assessing the potential impact of a new pathogen: how it may be detected and the extent of its host and geographic range. In Chapters 1 and 2, we outline two quantitative polymerase chain reaction (qPCR) diagnostic assays for *P. ramorum* and their use as part of a widespread survey, screening California plants for the pathogen. This research was instrumental in identifying *P.*

ramorum as an extreme generalist with a very large host range. Our detection protocol was adopted by the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA, APHIS) for screening potentially infected plant material for regulatory purposes (USDA, APHIS 2007).

As a better understanding of the pathogen and its impact on California plants developed, I shifted focus to the pathogen's most-susceptible and threatened host. The research outlined in Chapters 3 and 4 was aimed at collecting the information that will be necessary for developing evolutionary ecological hypotheses and management strategies for tanoak, which is speculated to face at least local extirpations (Hansen 2008, Manos et al. 2008). In Chapter 3, my colleagues and I report the first surveys of variation in susceptibility to *P. ramorum* on detached leaves of field-collected tanoak trees, with a corresponding analysis of variation and structuring in neutral genetic markers. We found significant differences among individuals that pointed to a low but non-zero heritability of susceptibility, and that the structure of phenotypic variation in this measure of susceptibility echoed that of the neutral markers. In both cases, there was low but significant variation among populations, with most of the differentiation residing within populations. There was no evidence of past selection on phenotypic differentiation. Environmental variation did play a role in susceptibility; we posited that given the low genetic and phenotypic differentiation among populations, environment is likely to play a large role in disease dynamics.

This survey of wild trees was a valuable first report, but it did not allow us to fully distinguish between genetic and environmental causes of variation in susceptibility. Tanoaks present a particular challenge to genetic studies, in that prior to the *P. ramorum* epidemic, they were not economically valued nor commercially propagated in large numbers. Consequently, no

individuals of known pedigree were available with which to calculate genetic parameters. In Chapter 4, I describe a project in which I, with my principal investigator Matteo Garbelotto, established a greenhouse common garden population of potted tanoak seedlings of known parentage concurrently with a field test garden in an infested natural area. The two gardens together provide a critical test population; laboratory assays allow us to form a much more complete picture of the various kinds of resistance to ramorum blight in tanoak, while the disease garden will validate and expand on laboratory studies. Using these study populations, we were able to calculate the heritability of susceptibility in detached leaves with much greater precision. The shadehouse garden allowed us to expand the assay techniques and types of host resistance we measured. In so doing we discovered highly variable responses of seedling families to intact-seedling tip inoculations, the majority of which variation was due to genetic factors.

The validity of a disease garden depends on the successful infection of study plants under natural conditions. Using the qPCR detection techniques described in Chapter 2, we confirmed seedlings' infection within the first 7 months of the garden's establishment, with disease incidence rising over the course of the study. Furthermore, we found that, though it was early in the timescale of a disease garden study, both *P. ramorum* infection status and family origin have significant effects on tanoak survival in natural disease conditions. Together with the laboratory assays, our findings show that tanoak does possess some forms of genetic resistance to *P. ramorum*. The gardens we established provide the infrastructure necessary to determine if this resistance affects survival under natural conditions, to further investigate its inheritance and action, and to test the effect of varying environment and host genotype on its expression.

Any management response to the progression of this emergent, destructive disease should inform and be informed by evolutionary ecological theory; the work presented here provides a

foundation on which to do just that. We found signs of heritable genetic variation in tanoak resistance to *P. ramorum*; such variation is a necessary prerequisite for evolution by selective force (Fisher 1930). Now, specific hypothesis concerning generalism, specialism, gene flow, and the types of host resistance may be tested using the gardens, populations, and assays we have developed. Tests of spatially explicit coevolutionary theories using forest trees are rare (Barbour 2009), the advance of *P. ramorum,* unfortunately, provides an opportunity to monitor plant-pathogen dynamics as they play out in real time.

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CHAPTER 1

Detection and quantification of *Phytophthora ramorum* from California forests using a real-time PCR assay[†]

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ABSTRACT

The timely and accurate detection of pathogens is a critical aid in the study of the epidemiology and biology of plant diseases. In the case of regulated organisms, the availability of a sensitive and reliable assay is essential when trying to achieve early detection of the pathogen. We developed and tested a real-time, nested PCR assay for the detection of *Phytophthora ramorum*, causal agent of sudden oak death. This technique was then implemented as part of a widespread environmental screen throughout California. The method here described is sensitive, detecting less than 12 fg of pathogen DNA, and is specific for *P. ramorum* when tested across 21 *Phytophthora* species. Hundreds of symptomatic samples from 33 sites in 14 California counties were assayed, resulting in the discovery of 10 new host species and 23 infested areas, including 4

new counties. With the exception of a single host, PCR-based discovery of new hosts and infested areas was always confirmed by traditional pathogen isolations and inoculation studies. Nonetheless, molecular diagnostics were key in early pathogen detection, and steered the direction of further research on this newly discovered and generalist *Phytophthora*.

INTRODUCTION

Phytophthora ramorum is the causal agent for the disease commonly called sudden oak death (SOD). While the disease is popularly known for its lethal, girdling cankers on susceptible oak species, the pathogen also causes less dramatic foliar symptoms on a wide—and ever-increasing—range of woody and herbaceous hosts (13,21,22,33). *P. ramorum* was first identified on European *Rhododendron* and *Viburnum* in 1993 (45), and then confirmed as the causal organism for SOD in California in 2000 (38). At the time of discovery, the known host list for *P. ramorum* in California included only two oak species, *Quercus agrifolia*, and *Q. kellogii*, and tanoak, *Lithocarpus densiflora*. Confirmed isolations of the pathogen originated exclusively from woodlands in Marin County, California. In Europe, confirmed isolations originated from a few horticultural nurseries in Germany and the Netherlands, on *Rhododendron* and *Viburnum* species (45). Since then, a widespread environmental screening in the United States has expanded the confirmed host list to 30 species in 12 California counties, and to Curry County in southern Oregon (43,44). Nurseries in many European countries are known to be infested (10,26,32,35). Infested nurseries have been reported in the United States in California, Washington State, Oregon, and in British Columbia, Canada (9). Infestations have been recently found in several states in the eastern U.S: for an update on the current situation see the USDA-APHIS Pest Detection and Management Programs Sudden Oak Death web page (44).

European and North American populations of the pathogen represent distinct lineages (4, 23), and are distinguishable by a range of phenotypic traits (4). European isolates tend to be faster growing, and are almost all of mating type A1 (4, but see 46). North American isolates have been observed to have broader sporangia and variable growth rates (D. Hüberli, personal communication), and populations in the wild have all been mating type A2 (D. Hüberli, personal communication). However, both A1 and A2 mating types have been reported in nurseries in Oregon, Washington, and British Columbia (19, 23).

Disease symptoms are reviewed in Davidson et al. (9). They include cankers, which often bleed viscous bark exudates, above the soil line in *Quercus* spp. and *L. densiflora* (38); and leaf blight, often associated with twig and branch dieback, in *L. densiflora* and all other hosts (15). *Quercus* species show trunk cankers alone, without foliar lesions. Species including maple (*Acer macrophyllum*), buckeye (*Aesculus californica*), and California bay laurel (*Umbellularia californica*) develop foliar lesions exclusively. Other species, including *Rhododendron*, manzanita (*Arctostaphylos manzanita*), and Pacific madrone (*Arbutus menziesii*), exhibit both foliar lesions and twig dieback. *L. densiflora*, in contrast, develops trunk cankers as well as both twig and foliar lesions (9). The disease has caused massive die-off of oaks and tanoaks in some areas, leaving up to 90% of adult tanoaks and 40% of adult coast live oaks dead in a stand (40). Species susceptible to foliar infections, rather than oaks, are now believed to be responsible for most of the sporulation and spread of the pathogen in nature. Bay laurel, for instance, is the plant species on which infection and sporulation is most abundant in California (15, 40). There is great concern about the potential devastating effect of *P. ramorum* if moved into non-infested areas. Greenhouse trials have shown that important plant species, native to other parts of the world, are highly susceptible to this pathogen (20,30,36,39,42). A range of nursery plants have

proven to be susceptible to infection by *P. ramorum* (39,45), and consequently, all transport of confirmed hosts from infested U.S. counties is now regulated (43,44). The effectiveness of this kind of regulation and quarantine depends on reliable detection of the pathogen.

Traditionally, morphological identification has been the preferred method for detection of pathogenic microbes. However, morphological identification can pose a number of difficulties. *Phytophthora* species, like many other microorganisms, may not always be culturable. Further, *Phytophthora* morphology may be plastic (3,11), and distinguishing characteristics can be subtle (12). *P. ramorum* has only been recently described (45), and much of its phenotypic variability may remain uncharacterized. Additionally, successful isolations require host-specific protocols. For example, leaves of Pacific madrone must be cultured within 40-60 days of infection, while tanoak leaves must be soaked in water for up to ten days before isolations may be obtained (D. Rizzo, unpublished data). The expanding host list, variable symptoms by host, plasticity in colony morphology, and variable success in culturing, result in the need for a sensitive and reliable method of detection that is not strictly dependent on our ability to isolate or morphologically identify the pathogen.

The polymerase chain reaction (PCR) has long been used to detect pathogens that may not be cultured, such as viruses (e.g., 47) and phytoplasmas (18,27). Taxon-specific PCR has been used as a method of screening for microbes such as mycorrhizal fungi (16) as well as plant pathogens including other *Phytophthora* species (2,24). Because specific primers are used to discern small amounts of microbial nucleic acids from a much larger quantity of host plant DNA, or because samples at times include PCR inhibitors, nested PCR may be required to detect low levels of infection (1,2,17).

Successful primer design for detection of a pathogen requires that the target region be: i.), unique to the organism of interest, and ii.), conserved across populations of the organism of interest. The internal transcribed spacer (ITS) region has been shown to be largely conserved within *Phytophthora* species, but differ across species (7,28). Phylogenies subsequent to that of Cooke et al. (7) based on alternate gene regions have largely upheld the groupings in the ITS-based phylogeny (23,31). Because the ITS sequence occurs in multiple copies in the genome, the target concentration is effectively increased, thereby increasing its value for diagnostic primers. Most importantly, sequence information is available in this region for nearly all known species of *Phytophthora* (7). The species most similar to *P. ramorum* in the ITS region are *P. lateralis* (differing by 11 base pairs) and *P. hibernalis* (differing by 39 base pairs). Consequently, we designed *P. ramorum*-specific primers within the ITS region.

The current technique has the further advantage of being able to be performed as real-time PCR, visualized using an intercalating dye such as SYBR® green. Real-time PCR allows products to be distinguished based not only on size but also on sequence, as melt temperatures will differ for same-sized but distinct products (37,41). Multiplex PCR with universal primers may be used as a positive control, to indicate whether an observed negative is a true PCR-negative, or is the result of a failed extraction (13,50).

An objective of the study was to develop a nested PCR-based assay to detect *P. ramorum* from DNA extracted directly from symptomatic tissue of putative hosts. The assay was used in a statewide survey to identify new infested areas as well as new plant hosts. The results reported here include information gleaned from that survey, as well as data on the PCR assay's specificity, sensitivity by host substrate and seasonality, and potential use for pathogen DNA quantification.

MATERIALS AND METHODS

Statewide Disease Survey

The isolation of *P. ramorum* in July 2000 from dying tanoak and oaks in central California (38) prompted the beginning of a statewide survey to determine the extent of the distribution of the pathogen. The survey, still ongoing in 2004, covers the entire geographic range of woodlands in California. It was initiated before the formal description of the pathogen, and at a time when our understanding of disease symptoms and of the pathogen host range was extremely limited. Thus, sites were examined not only for the presence of known *P. ramorum* symptoms on confirmed hosts, but also for the presence of unusual disease symptoms on any plant species. At each survey site, symptoms were described, locations of symptomatic trees were mapped, and symptomatic tissue was collected for cultural and PCR-based diagnoses, as described below. Symptomatic tissue was cultured by plating a small section from the margins of leaf/branch lesions or stem cankers on PARP selective medium (per liter: 17 g corn meal agar, 0.25 g Ampicillin, 0.4 ml of 2.5% Pimaricin, 0.01 g Rifampicin in 1 ml DMSO, 5 ml of 0.5% PCNB in ethanol). For DNA analyses, a comparable portion of symptomatic tissue was sealed in plastic bags or polypropylene tubes and stored at 10°C for a maximum of four days. Samples were then excised from the margins of necrotic areas with a scalpel and transferred to a 2.0 ml polypropylene tube, frozen, and lyophilized.

At times, diagnosis was ambiguous, e.g. no *P. ramorum* culture could be obtained from symptomatic plant tissue that had resulted positive based on the PCR-based technique described here. In these cases, plants were sampled again and isolations were repeated, often modifying the

original isolation techniques either by pretreatment of the tissue, such as by soaking the sample for an extended period of time, or by better selection of the tissue to be plated.

An excess of 2000 samples were processed in the course of the survey, by PCR-based methods and by culturing. Because the PCR protocol changed during the course of the survey, and because PCR-based results were consistently in agreement with those obtained through culturing, with a few exceptions (see Results), here we report only on samples collected between May 2001 and August 2002. Samples collected within this time frame were all processed according to the methods described in this paper.

The frequency of successful *P. ramorum* detection was determined for a total of 528 samples, comprising all plant substrates tested in the survey. To take into account the effect of season, samples were grouped by collection date according to rainfall records into three categories, which represent distinct climatic periods: March-June (intermediate rainfall, following months of heavy rains, warm temperatures); July-October (little or no precipitation, hot temperatures); and November-February (heavy rains following the dry season, cold temperatures) (5,6). Based on the current understanding of the biology of *P. ramorum,* prolonged rain events and warm temperatures like those normally recorded in the March-June period are optimal for infection and spread of the pathogen (40).

In order to compare efficacy of diagnosis by either culturing or PCR, a subset of 216 samples for which no culture was initially obtained was further screened by the PCR-based method described here, as well as by an additional round of isolations. Results of this analysis were also grouped by substrate, to compare the efficacy of the PCR method on samples taken from bay laurel leaves, leaves from hosts other than bay laurel, oak and tanoak wood.

DNA extraction

Bulk DNA was extracted from lyophilized tissue using a modified CTAB extraction: Lyophilized tissue was pulverized with glass beads in a FastPrep® instrument (Bio101, Carlsbad, CA) for 5 to 30 s at 4,000 rpm. Pulverized tissue was subjected to two repetitions of freezing (on dry ice for 2 min) and thawing (at 75°C for 2 min) in 350 µl CTAB. DNA was purified in phenol:chloroform:isoamyl alcohol (25:24:1), further cleaned by using the Geneclean® Turbo Nucleic Acid Purification kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions, and eluted in 30 µl ultra-pure water (nano-purified, autoclaved, and UV-irradiated). DNA extracts were stored at –20°C in 1/10 TE buffer. Extracts from mycelia were diluted 1/1000 in ultra-pure water prior to amplification, while extracts from plants were diluted 1/100.

Development of primers and PCR conditions

Two sets of specific primers (Table 1) were designed based on a manual revision of the *Phytophthora* species ITS alignment of Cooke et al. (7) to include *P. ramorum*. Primer3 v. 0.6 software (available online from Whitehead Institute for Biomedical Research, Cambridge, MA) was used to select sites that were unique to *P. ramorum*, paying extra care to situate polymorphisms between *P. ramorum* and *P. lateralis* at the 3' end of the Phyto1 and Phyto4 primers. Phyto2 and Phyto3 were designed internal to Phyto1/Phyto4; this pair is also specific to *P. ramorum*. Universal primers were designed in the conserved region of the 28S rDNA sequence.

The first amplification was performed using primer set Phyto1/Phyto4. An aliquot of 6.25 µl of diluted bulk DNA was included in each 25.00 ml PCR reaction (1X reaction buffer

(Invitrogen, Carlsbad, CA), 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.5 μM each primer, 1.25U Platinum *Taq* Polymerase (Invitrogen, Carlsbad, CA)). Amplifications were carried out in an iCycler thermalcycler (Bio-Rad, Hercules, CA) under the following conditions: denature at 94°C for 1min 25 s; then 34 cycles of denaturing at 93°C for 35 s, anneal at 62°C for 55 s, extend at 72°C for 50 s, adding 5 s at each cycle; a final extension at 72°C for 10 min. Ramp rate: 3.3°C/s heating, 2.0°C/s cooling.

Products from the first amplification were diluted 1/500 in ultra-pure water, then subjected to the second amplification using primer set Phyto2 /Phyto3, using either a real-time or convential PCR method. For real-time amplification, an aliquot of 6.25 ml of each dilution was included in each 25.00 ml reaction (1X reaction buffer (Invitrogen, Carlsbad, CA), 0.2 mM each dNTP, 3.0 mM $MgCl_2$, 10^{-5}X SYBR® green (Sigma, St. Louis, MO), 10^{-5}X flourescein (Bio-Rad, Hercules, CA), 0.5 μM each primer, 0.75U Platinum *Taq* Polymerase (Invitrogen, Carlsbad, CA).

Real-time second round amplifications were carried out in an iCycler IQ real-time capable thermalcycler (Bio-Rad, Hercules, CA), using the protocol as described for first-round amplification. Real-time data were collected during the extension step. Immediately following amplification, product melt temperatures (T_m) were determined with the following conditions: 110 cycles at 62°C for 10 s adding 0.3°C at each cycle (melt curve, data collection step). Ramp rate: 3.3°C/s heating, 2.0°C/s cooling. Unless otherwise specified, this real-time, nested protocol was used in all tests here described.

The protocol for the second round of conventional PCR was as for real-time, but with reaction reagents and conditions exactly the same as for the Phyto1/Phyto4 amplification.

Products were visualized using agarose gel electrophoresis (1.3 to 1.5 % agarose in 0.5X TBE buffer).

Positive and negative controls

To ascertain DNA extractions were successful, universal primers were developed in the 28S region of the rDNA, 1909 bp from Phyto1/Phyto4 amplicon. To determine the expected fragment size, the primers were aligned against all available complete large subunit rDNA sequences for organisms within the same genera as the pathogen and its known hosts: *P. megasperma* (GenBank X75631.1), *Quercus suber* (GenBank AY428812.1), and *Aesculus pavia* (GenBank AF479138.1). It was confirmed that this primer set could amplify its target simultaneously with the Phyto1/Phyto4 and Phyto2/Phyto3 primers, without interference (data not shown). Reaction parameters for both first and second rounds are as previously described, with the addition of 0.5 mM of each 28S primer to both the first and second rounds of amplification.

A minimum of one negative water control was included with each PCR run. Ultra-pure water was added to the reaction well in place of DNA template before the first amplification. Afterwards, this control was treated identically to the unknown samples; it was diluted, and then subjected to the second amplification. For the environmental screen, each 96-well plate included a minimum of 16 such water controls. Any plate in which positive results were observed in a water control was considered contaminated, and the results discarded.

Several precautions were taken to avoid DNA contamination of plant samples and PCR reactions. All amplified products were spatially segregated from other laboratory functions, and never introduced into the area in which first-round PCR was prepared. All first-round PCR

reactions were set up in either a laminar flow hood (cleaned thoroughly with DNA-Away (Molecular BioProducts Inc., San Diego, CA) before use) or a plexiglass box that had been subjected to 15 minutes of germicidal UV-radiation. Second-round PCR-cocktails were prepared and aliquotted in the laminar flow hood or the plexiglass box, then removed to the high-DNA area before template was added. Separate sets of pipettes were maintained for amplified products, low-concentration DNA, and DNA-free applications. Aerosol-resistant pipette tips were used at all times.

Amplicons from twenty-six plant samples were sequenced to confirm pathogen identity, including an isolate from each newly discovered host. PCR products (obtained through conventional nested PCR as outlined above) were cleaned via QiaQuick PCR Purification kit (Qiagen, Qiagen Sciences, MD), as per kit instructions, except that cleaned products were eluted in 30 μl salt-free water. Cleaned products were cycle-sequenced with 4 μl Big Dye Terminator v3.0 (Applied Biosystems, Foster City, CA), 2.4 pmol salt-free primer and 5-20 ng DNA (template concentration determined by gel). Cycle-Sequencing was performed on a thermalcycler (Bio-Rad iCycler, Hercules, CA) according to ABI-recommended protocol. Samples were de-salted in ethanol as per ABI instructions (ABI Prism 3100 Genetic Analyzer Sequencing Chemistry Guide). Samples were brought up in 10μl Hi-Di Formimide (Applied Biosystems, Foster City, CA) and denatured on a thermocycler (95˚C for 5 min, followed by a hold at 4˚C). Capillary electrophoresis was performed on an ABI Prism 3100 Genetic Analyzer, with POP-4 polymer on a 36cm capillary array, and data were collected on Data Collection Software v1.0 and analyzed with Sequencher v.4.1.2 (Gene Codes Corporation, Ann Arbor, MI).

Season and plant substrate

To corroborate the apparent effects of plant substrates and season on detection frequency of *P. ramorum*, the PCR assay was run on a total of 424 samples, all additional to samples included in the disease survey. All 424 were obtained from plants from which *P. ramorum* had previously been isolated by culture.

In order to investigate the effect of season on the PCR-based diagnostic assay, 299 samples of exudates from bark cankers on oak and tanoak in Santa Cruz County, CA were collected periodically from March 2001 until February 2002. *P. ramorum* had been successfully isolated from cambium taken from the cankers on each of the selected trees. Exudates were processed as described above for plant tissue, and assayed both by PCR and culturing. Results were compared among collections obtained during the three seasonal categories described above.

PCR assays to compare efficacy on different host substrates were performed on a subset of 204 samples. The substrates tested included leaves from bay laurel, leaves from hosts other than bay laurel, oak wood, and exudates from bark cankers. Collections and PCR assays were all performed in what we understand to be the most favorable period of the year for detection of *P. ramorum* (March-June). Results from 2001 and 2002 were pooled for this analysis.

Specificity and sensitivity

Cultures of *P. ramorum* and of 20 additional *Phytophthora* species (Table 2), were grown in either potato dextrose or pea broth for 7-10 days, then filtered and lyophilized. The DNA of three *P. cambivora* isolates (NY217, 444, and NY249) was extracted from mycelia taken from 110 mm rye agar plates. The plates were microwaved on 50% power for 30 s with the cover on.

The mycelia were then blotted on clean paper towels, transferred back to the original plate, and microwaved on 50% power for an additional 6 to 8 s. The mycelia were blotted again on clean paper towels until all melted agar was removed, then frozen and lyophilized.

Specificity of amplification was tested on DNA extracted from the pure cultures. Extracts were diluted 1/1000 in ultra-pure water before being amplified. To insure the presence of DNA, all extracts had been successfully amplified using the universal primers ITS1/ITS4 (Table 1) (48).

The specificity of amplicon melt temperature was determined in a series of steps. First, six replicates of pure-culture DNA extracts from 18 isolates of 15 species (Table 2) were amplified with the universal primers ITS1/ITS4 (parameters as described for Phyto1/Phyto4 first round amplification). Products were then diluted 1:500 and amplified with the primer set Phyto2/Phyto3 under relaxed conditions, in order to allow amplification of sequences that did not exactly match the primers. Parameters were as previously described, except that the annealing temperature at 56°C, and $MgCl_2$ was at 3.5 mM. Melt temperatures (T_m) were compared among all successful amplicons using Tukey's HSD test with alpha at 0.05 (JMP v.5.01, SAS Institute, Cary NC).

A minimum DNA detection threshold was determined for *P. ramorum* and *P. lateralis* by testing a ten-fold dilution series of known DNA concentration. DNA extracts were initially tested at concentrations on the order of 10 ng per µl; the concentration was decreased until no products could be observed by either real-time PCR or agarose gels. To compare sensitivity of the nested protocol, as opposed to amplification by Phyto1/4 alone, a subset of plant samples were screened with nested PCR (real-time protocol). A total of 159 samples of exudates, foliar and wood tissue from symptomatic plants putatively infected with *P. ramorum*, were included in the comparative

analysis. Products were visualized with agarose gels after both first and second rounds. In addition, the final products were visualized with real-time PCR. Results from the full-nested assay were compared to those from the single Phyto1/Phyto4 run.

Quantification of pathogen DNA

Real-time PCR can be used to quantify starting concentrations of DNA by the threshold cycle (Ct), the amplification cycle at which the concentration of DNA produce in the PCR reaction climbs above a baseline, as visualized by flourescence (51). To determine whether Ct corresponded to viability, symptomatic leaves of *U. californica* were collected from Big Sur, CA in April and May 2002. Leaf pieces were plated on PARP selective media, and the remaining leaf tissue was frozen. Bulk DNA was extracted from the tissue as described above, and the samples were screened for *P. ramorum* using the nested, real-time protocol. All samples positive for *P. ramorum* were tested again in a single 96-well plate (N=51), to allow for comparison of Ct among samples. First round amplifications were duplicated using real-time PCR with SYBR® green to allow for the determination of Ct and to ascertain that first round products were still in the linear growth phase when amplification was terminated—a prerequisite for quantification with nested real-time PCR (34). Fluorescence baseline and threshold values for the second amplification round were determined automatically, and all wells confirmed positive by a T_m in the expected range. Threshold cycles were compared for samples with positive isolations (N = 21) versus samples from which a culture of *P. ramorum* could not be obtained (N = 30) using Student's t-test. Prediction intervals were calculated by hand (52).

RESULTS

Statewide disease survey

In the widespread survey of plants suspected of *P. ramorum* infection, we detected infection in 204 out of 528 samples tested using the assay presented here. We detected 10 new hosts (Table 3) and 4 new infected counties (Contra Costa, Humboldt, Mendocino and Solano counties) using this method. Twenty-three of 33 sites in 14 counties were found to be positive for *P. ramorum* during the exploratory surveys of California. Melt curves of amplified products were used to determine results (mean T_m 90.8°C; range 89.9°C to 91.7°C; s.d. 0.3°C). One or more amplicons from each new host were sequenced; all had complete homology to the *P. ramorum* sequence.

The method developed detected *P. ramorum* infection in a number of samples where culturing failed (Figs. 1 and 2, Table 3). PCR results were in accordance with cultural results in 169 of the 216 samples for which the two techniques were compared. For any subset of samples, PCR detection was always more sensitive than direct culturing, although a few individual isolates were positive by culturing and negative by PCR (47 samples were positive by PCR detection but not by culturing, versus 4 positive by culturing but not by PCR). In addition, the PCR-based assay detected *P. ramorum* in 72 of the 299 canker exudates processed (Fig 3). In contrast, no cultures were isolated from the exudates, even though *P. ramorum* had been isolated from wood tissue within the cankers themselves.

Season and plant substrate

Inspection of the detection frequency in *Quercus* spp. and *L. densiflora* canker exudates by the nested PCR technique reveals that detection was most successful during the months of March through June, when California's Mediterranean climate is warm, yet the rains have not yet stopped for the summer (Fig 3). This pattern was also observed in detection frequencies of *P. ramorum* throughout the environmental survey; our highest detection frequency from symptomatic foliar and wood tissue was during the months of March through June, when 50% of samples tested were positive. November through February, and July through August, each showed a lower detection frequency, at 36% and 34%, respectively.

Detection levels varied by host: leaves of *U. californica* had the highest frequency based on both PCR and culture detections methods. Wood samples from *Quercus* spp. and *L. densiflora*, and leaves of hosts other than *U. californica* had an intermediate rate of detection by both methods (Fig.2). The finding that frequency of successful diagnosis is highly dependent on host and substrate was mirrored by the results of the analysis shown in Figure 4. In this case samples representative of different substrates were all collected in the spring, the most favorable season for detection of *P. ramorum*. Samples were all known to be infected by the pathogen, as they came from individual trees or plants from which *P. ramorum* had been previously isolated. Again, leaves of *U. californica* represented the substrate with the highest level of detection, while canker exudates from *Q. agrifolia* and *L. densiflora* had the lowest success rate by PCR and were never culturable, even though *P. ramorum* had been isolated from tissue collected from the cankers themselves. (Fig. 4).

Specificity and sensitivity

P. lateralis was the only other species among the 20 tested to cross-amplify in the assay. This occurred only at high DNA concentrations (Table 2). In contrast, *P. ramorum* could be detected at concentrations as low as 12 fg. *P. cambivora* has previously been reported to be cross-amplified by this primer set (9). However, sequencing revealed the product of the isolate previously amplified by our lab to be the result of cross-contamination, as the amplified sequence was a complete match for *P. ramorum*. A new extraction of this isolate was not amplified in repeated tests, nor were the other four *P. cambivora* isolates tested (Table 2). We hypothesize that the original culture, which was isolated from *Q. agrifolia* suspected of harboring *P. ramorum*, was actually mixed, and contained both pathogens. If this original culture had been predominantly *P. cambivora* (as it was morphologically identified), the traces of *P. ramorum* may have been removed as the culture was sequentially passed through plates.

Four *Phytophthora* species were amplified by Phyto2/Phyto3, from ITS1/ITS4 amplicons (note that this does not represent cross-reactivity of our diagnostic method; the first round primer set is different and PCR conditions were relaxed to enhance reactivity). Of these, *P. lateralis* and *P. erythroseptica* were indistinguishable from *P. ramorum* by T_m (*P. ramorum* mean T_m 90.3°C, s.d. 0.21°C; *P. lateralis* mean T_m 90.0°C, s.d. 0.31°C; *P. erythroseptica* mean T_m 90.4°C, s.d. 0.35°C), but *P. hibernalis* had a significantly lower T_m (mean T_m 88.8°C, s.d. 0.25°), and *P. cryptogea*'s was significantly higher (mean T_m 91.0°C, s.d. 0.12°C).

Detection of *P. ramorum* in the 159 plant samples of unknown infection status increased from 23, in the first round of PCR, to 77, after both rounds. Positives as determined by agarose gel electrophoresis after the final PCR were all identical to those as determined by product melt curves through real-time PCR.

Quantification of pathogen DNA

In testing threshold cycle count (Ct) by viability, culture-positive symptomatic samples had lower Ct values than did culture-negative samples (culture-positive: mean Ct 13.15, s.d 3.71, range 1.6 to 17.3; culture-negative: mean Ct 15.43, s.d. 3.71, range 9.5 to 22.6; t-test, P = 0.033). Variances were equal in each group (O'Brien test, P = 0.89). The prediction interval for culture positive samples was 5 to 21, compared to 8 to 23 for culture negative samples.

DISCUSSION

The method presented here was instrumental in the early stages of research on sudden oak death in California. This technique allowed us to confirm *P. ramorum* infection in over 204 symptomatic plant samples from 33 California sites, and allowed us to expand the confirmed host range of *P. ramorum* by 10 hosts and 4 California counties far sooner than if identification were based on pathogen isolation alone. These surveys were designed to rapidly provide information on overall distribution of the newly discovered pathogen. Results from PCR analyses accelerated our understanding of the wide range of symptoms caused by this pathogen. This knowledge in turn, accelerated our discovery of new hosts, by broadening the aim of our collections to include plants displaying novel symptoms.

This was one of the first studies where basic information, such as host and geographic range, of a new forest disease has been discovered using molecular-based diagnostics. The method described here is sensitive, and the limited cross-reactivity is not expected to interfere with the accuracy of diagnoses from environmental samples. The amount of *P. lateralis* DNA

required for amplification is relatively high; in addition, *P. lateralis* has a very limited host range, encompassing only two known species, neither of which is a known host for *P. ramorum*.

The high sensitivity of the technique is in part due to the presence of a large number of copies of ribosomal DNA within each cell. Methods based on molecules not as abundant in the genome will inevitably result in decreased sensitivity. The results indicate *P. ramorum* DNA concentration in most plants is low and sensitivity is a critical diagnostic issue. Our nested approach based on the multicopy rDNA provides a viable solution. It is common for diagnostic PCR to require a nested design (1,2). While using nested PCR does open the technique to greater risk of false positive results due to contamination, proper laboratory techniques minimize risk. To maximize detection of false positives due to DNA contamination, we suggest running a number of negative samples equal to the number of positive samples. Negatives, consisting of plant tissue not infected with *P. ramorum*, must be carried through the entire process, including tissue preparation, DNA extraction, dilution, and PCR reactions. Independent corroboration by separate labs should be used to verify critical results. We report here only results from sites from which repeated positive results were obtained, and were eventually confirmed by isolation of the pathogen. Occasional, unique PCR-positives from any given site (i.e., could not be obtained from that site again) were conservatively assumed to be due to laboratory contamination and disregarded. Any given positive result should be able to be replicated by re-processing the original extracted DNA, and ideally, re-extracting tissue from the same host plant. The latter poses problems, however, if the second sampling is temporally removed from the first. Low frequencies of true, positive results may disappear if a site is later sampled at a time less favorable for pathogen growth.

The multiplex reaction using the Phyto primer sets and the 28S primer pair allows differentiation between true negatives (28S amplicon present, Phyto2/3 amplicon absent) and negatives due to failed extractions (28S and Phyto2/3 amplicons absent) (Fig. 1). In our experience, PCR false negatives can result when plant material is processed improperly or DNA extract and first round amplifications are not stored at constant low temperatures (less than -20°C).

This study provides some of the first data on seasonality of molecular detection success for *P. ramorum*. The higher detection frequency from March through June is likely due to increased pathogen growth and increased sporulation and infection rates during warm, wet conditions (8,15). The data suggest that surveys for *P. ramorum* are best carried out in late spring, as surveys during the late summer months are likely to underestimate disease abundance, even in known infestation areas.

Two analyses presented in this study (Figs. 2 and 4) indicated the efficacy of diagnosis both by culturing and PCR was different when dealing with different plant species and substrates. Thus, it seems wise to test the potential sensitivity of the diagnostic protocols each time a new host or substrate need to be assayed, rather than inferring it from results obtained on previously assayed hosts and substrates.

We further describe here a novel non-destructive way of sampling infected oak and tanoak trees. Successful diagnoses were obtained by processing samples of the viscous bleeding caused by *P. ramorum* on oak and tanoak trunks, by collecting the sap on the bark surface without wounding the tree. In contrast, the pathogen was not isolated by culture from any of the exudates, though *P. ramorum* had previously been isolated from wood within the cankers themselves. Non-wounding assays are essential for long-term studies, as the wounds themselves

can artificially alter the dynamics of a forest stand by being an infection court for other pathogens or an attraction to insects.

The results of this study indicated that in the case of "difficult" samples not yielding viable cultures on the first isolation attempt, PCR-based diagnosis was more sensitive than a second isolation attempt (Fig.2). The use of the molecular assay here described significantly reduced the number of false negative samples: these are samples that based on culturing alone would have been regarded as "non-infected". The nested PCR assay used in this survey is thus a much more sensitive assay than culturing alone, and its use will improve our ability to detect the pathogen, and hopefully help us to restrict its movement.

As described elsewhere (14,39) in the absence of successful culturing, PCR-based detection was an integral component in the determination of a new species as a host for *P. ramorum*. The presence of characteristic disease symptoms on a new putative host, multiple independent PCR positives confirmed by sequencing of the amplicon, and successful inoculation studies using isolates from another host, were taken as evidence *P. ramorum* was causing disease on the host in question. It should be noted that in all cases but one (i.e. manazanita spp.), isolates of *P. ramorum* were eventually obtained from the plant species in questions. Subsequently, Koch's postulates were completed to confirm these species as hosts (15,39).

SYBR® green-mediated real time PCR, including melting temperature T_m analysis of the final amplicon, does not require gel electrophoresis. It is thus faster and more accurate than regular PCR, as melting curves can discriminate among some equally sized amplicons of different sequence. Furthermore, real-time PCR allows for the quantification of the pathogen DNA (49,50,51). Provided that samples in the first PCR round are still in linear growth phase (which may require a reduction in the number of cycles of the first round), the nested technique

can also provide some quantification data (34). It may be possible then, to distinguish hosts with active, growing infections by the quantity of target DNA present in a given sample. A lower threshold cycle count (Ct) corresponds to higher DNA concentration in the starting sample. It was observed that viable samples had a lower mean PCR threshold cycle than samples from which cultures were not obtained, and more importantly, that the ranges and prediction intervals of Ct for culture-positive and culture-negative samples were distinguishable. Thus, if the Ct value of an additional tested sample falls in a non-overlapping area of the prediction interval, it may be possible to predict whether the sample has a viable infection. For the dataset presented, any additional samples with a Ct falling below 8 would be predicted to be viable, while sample with Ct's above 21 would be predicted to be non-viable. Any sample falling between 8 and 21, i.e., the interval in which the range of viable samples and samples from which cultures were not obtained overlap, would be of undetermined viability. It should be noted that for this method to have predictive value, sample sizes must be sufficiently large, and representative of the target population. The prediction interval is a more conservative marker than the range, because it takes into account uncertainty of where any new data points are likely to fall—extending the area of overlap between culture-positive and culture-negative results.

The method described in this paper has the advantage of being available to laboratories without real-time PCR capacity. Rather than using SYBR® and melting temperatures of final amplicons, products can be analyzed through agarose electrophoresis. The technique is equally sensitive for both the European and the North American populations of the pathogen (25, 29). The broad applicability of this technique makes it a valid and useful diagnostic tool. The method has been evaluated in the U.S. by USDA-APHIS and by the Dutch Plant Protection agency, and

has been approved both in the USA and in the Netherlands as a diagnostic tool to be used by the respective regulatory agencies (25,29).

In conclusion, this method of detection has proven to be effective in the field for the study of an emerging plant disease. The PCR assay described has been responsible for the discovery of new hosts and infested counties, sometimes months before the pathogen was successfully isolated from those areas. This protocol has been of critical importance in developing our understanding of sudden oak death in California and shows that an integrated approach using traditional plant pathology and molecular diagnostics is essential when studying a new pathogen causing a variety of symptoms on a broad range of hosts.

ACKNOWLEDGEMENTS

We thank C. Blomquist, M. Coffey, L. Marais, and P. Tooley for providing cultures, and D. Chambers and S. Tjosvold for exudate samples. K. Ivors contributed DNA extracts, sequences, and helpful discussion. We thank J. Davidson, T. Harnik, M. Mejía-Chang, G. Slaughter, and M. A. Smith for technical assistance, and J. Correll, R. Warner, and two anonymous reviewers for helpful comments. This research has been supported by grants from the Gordon and Betty Moore Foundation, the California Department of Food and Agriculture, and the USDA Forest Service, Pacific Southwest Region.

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Table 1. Sequences of primers used for amplification of *P. ramorum* and hosts.

Name	Sequence 5′ to 3′	Amplicon size (bp)
Phyto1	CATGGCGAGCGCTTGA	687
Phyto4	GAAGCCGCCAACACAAG	
Phyto2	AAAGCCAAGCCCTGCAC	291
Phyto3	GGTGGATGGGGACGTG	
ITS1[a]	TCCGTAGGTGAACCTGCGG	917
ITS4[a]	TCCTCCGCTTATTGATATGC	
28SF	GGAACGTGAGCTGGGTTTAG	194[b]
28SR	TTCTGACTTAGAGGCGTTCAG	191[c]

[a]From White et al. (48)
[b]For *Phytophthora ramorum*
[c]For plant hosts

Table 2. Isolates of *Phytophthora* species used to determine specificity of reaction. Unless otherwise noted, cultures are from the collection of D. Rizzo. Isolates marked with an asterisk were used in the study of Phyto2/3 amplicon melting temperature.

Species	Local isolate no.	Alt isolate no.	Host	Origin	Lowest concentration amplified (ng DNA/25 ml reaction)
Phytophthora boehmeriae^{PT}	MP2	325	*Boehmeria nivia*	Papua New Guinea	Not cross-amplified
P. cactorum^{PT}*	MP19	311	*Pseudotsuga menziesii*	Washington	Not cross-amplified
P. cambivora^{PT}*	MP14	P198513	*Quercus agrifolia*	California	Not cross-amplified***
P. cambivora^{PT}*	MP22	444 (A2)	*Prunus dulcis*	California	Not cross-amplified
P. cambivora^{PT}	MP23	443 (A1)	*Prunus avium*	California	Not cross-amplified
P. cambivora^{PT}*	NY217		*Malus domestica*	New York	Not cross-amplified
P. cambivora^{PT}*	NY249		*Malus domestica*	Oregon	Not cross-amplified
P. capsici^{PT}*	MP26	302	*Capsicum annuum*	Florida	Not cross-amplified
P. cinnamomi^{MC}*	P6379 (A1)		*Ananas comosus*	Taiwan	Not cross-amplified
P. citricola^{LM}*	MP18			California	Not cross-amplified
P. cryptogea^{PT}*	MP11	438	*Lycopersicon esculentum*		Not cross-amplified
P. drechsleri^{LM}					Not cross-amplified
P. erythroseptica^{PT}*	MP6	355	*Solanum tuberosum*	Maine	Not cross-amplified
P. gonapodyides^{PT}*		393	*Malus domestica*	New York	Not cross-amplified
*P. hibernalis**	1895		*Aquilegia vulgaris*	New Zealand	Not cross-amplified
P. hibernalis	1896		*Citrus sinensus*	Portugal	Not cross-amplified
P. nemorosa	MP16			California	Not cross-amplified
P. infestans^{LM}					Not cross-amplified
P. lateralis	91/9/9-1		*Chamaecyparis lawsoniana*	California	2.4
*P. lateralis**	PL33		*Chamaecyparis lawsoniana*	California	0.7
P. megasperma^{PT}*	MP10	309	*Pseudotsuga menziesii*	Washington	Not cross-amplified
P. megasperma f.sp. *glycinea*^{PT}	MP20	312	*Glycine max*	Wisconsin	Not cross-amplified

P. palmivora[PT]*	MP8	427	*Theobroma cacao*		Not cross-amplified
P. parasitica[PT]*	MP3	331	*Nicotiana tabacum*	North Carolina	Not cross-amplified
P. pseudotsugae[PT]	308	308	*Pseudotsuga menziesii*	Oregon	Not cross-amplified
*P. pseudosyringae**	P40		*Quercus agrifolia*	California	Not cross-amplified
P. syringae[CB]	MP15	P115773A	*Rhododendron spp.*	California	Not cross-amplified
*P. ramorum**	Pt102		*Quercus agrifolia*	California	1.29 E-6 (12.9 fg)

[PT] Courtesy of Paul Tooley, USDA ARS, Fort Detrick, MD
[CB] Courtesy of Cheryl Blomquist, California Department of Food and Agriculture, Sacramento, CA
[MC]Courtesy of Mike Coffey, University of California, Riverside, CA
[LM]Courtesy of Laurence Marais, University of California, Riverside, CA

CHAPTER 2

TaqMan chemistry for *Phytophthora ramorum* detection and quantification, with a comparison of diagnostic methods

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Previously published as *Phytopathology* 2006, vol. 96, issue 8, pp. 846-854

ABSTRACT

The choice of detection method for phytopathogens can be critically important in determining the success or failure of pest regulation systems. We present an assay for *Phytophthora ramorum* that uses 5' fluorogenic exonuclease (TaqMan) chemistry to detect and quantify the pathogen from diseased tissue, and include a universal primer and probe set for an internal positive control. This method is sensitive, detecting as little as 15 fg of target DNA when used in a nested design, or 50 fg when used in a single round of polymerase chain reaction. None of the 18 other *Phytophthora* species tested was amplified by this assay. A comparison of the nested and non-

[1] Responsibility was as follows: K. Hayden, quantitative PCR; K. Ivors, development of PCR primers and fluorogenic probes; C. Wilkinson, comparative studies; M. Garbelotto, principal investigator.

nested TaqMan assays, and of one other nested assay, showed nested methods to be significantly more sensitive than non-nested, and that host substrate significantly affected sensitivity of all assays. The nested TaqMan protocol was successfully field-tested; *P. ramorum* was detected in 255 of 874 plants in California woodlands, while the single-round TaqMan protocol detected significantly fewer positive samples. Finally, we documented increases in the quantity of pathogen DNA in *U. californica* leaves in initial stages of infection.

Keywords: forest pathology, PCR inhibition, quantitative PCR, real-time PCR, sudden oak death

INTRODUCTION

Phytophthora ramorum is a recently discovered oomycete plant pathogen, which causes the disease known as sudden oak death. The disease's common name arises from its symptoms on *Quercus* species and *Lithocarpus densiflora*, where it causes girdling trunk lesions that often result in rapid death of the host (29). This pathogen also causes stem and leaf lesions on a wide range of other hosts (9), and has been found in an expanding range of nurseries and wildlands throughout the United States and Europe (2,19,28,42)

Detection of the pathogen in plant material is a critical problem for scientists and regulators concerned with stemming the expansion of this emerging disease. Since Werres *et al.* (36) formally described the pathogen, multiple molecular detection methods have been published (12,20,23,33), or are in preparation (5,7). Molecular methods of detection have often been preferred because diagnosis by direct isolation from symptomatic plant tissue is complicated and can be dependent on numerous factors, including inadequate sample storage and unfavorable environmental conditions during the time of sampling, which can lead to false-negative

isolations and misdiagnosis of infected plant material (4,6,10,17). Real-time PCR methods can be used to detect the organism of interest, as well as to quantify the organism's DNA (e.g., 21,31,32,34). The 5' fluorogenic exonuclease (TaqMan) assay has been broadly used to develop assays with enhanced target specificity. Enhanced specificity is achieved because the DNA sequence of the target organism must be matched not only by the two primers, but also by an internal probe, usually 15-40 bp long (13).

TaqMan chemistry can be used with confidence to quantify the extent of pathogen colonization of host tissue, if quantity of pathogen DNA is expressed in relation to total plant DNA (1,39,40). This quantitative capability may then be used to distinguish classes of partially-resistant host cultivars, characterize stand-level colonization, or otherwise increase understanding of host-pathogen relationships at the molecular scale. While other real-time PCR assays, including those using TaqMan chemistry, have been developed for *P. ramorum* (5,7,12,33), to our knowledge none has fully developed the mthods' quantitative capabilities for tracking pathogen colonization.

The foremost objective of sample diagnosis is to reliably detect the pathogen in host tissue. Nested PCR is required for detection of *P. ramorum* when the amount of pathogen material is very small, or when inhibitors are present in host tissue extracts (12,23). While methods of detecting *P. ramorum* have proliferated, only limited comparative data are available to evaluate sensitivity of TaqMan vs. non-TaqMan, and of nested vs. single-round approaches. Although sensitivities can be theoretically calculated and tested using DNA isolated from pure culture, the substrate from which detection is attempted can introduce enormous variability in assay sensitivity (23,35,37). This variability demands direct comparisons of available assays when their purpose is to detect the pathogen directly from host tissue.

The objectives of this study were: (i) to develop a reliable, quantitative method for detecting *P. ramorum* in plant tissue, (ii) to compare the sensitivity of this method to two others currently available, (iii) to use the assay to detect the pathogen in samples collected from California woodlands, and (iv) to determine if this method can be used to quantify the pathogen DNA in infected plant tissue.

MATERIALS AND METHODS

Assay specifications

Phytophthora ramorum specific primers (Pram5 and Pram6) and an internal dual-labeled fluorogenic (TaqMan) probe (Pram7, Table 1, Fig. 1) were designed within the internal transcribed spacer region 2 (ITS2) of the rDNA cluster, using the software Primer3 (30). These primers and probe were designed to lie internally to the region amplified by another *P. ramorum* specific primer set, Phyto1 and Phyto4 (12, Table 1, Fig. 1). As such, sensitivity may be improved by using the set Phyto1/Phyto4 in a first-round, or pre-amplification, step prior to amplification with TaqMan chemistry.

To control for successful DNA extraction, an additional universal primer set and TaqMan probe were developed in a conserved section of the small subunit of the rDNA (Lt1UnivPrimer, RtUnivPrimer, UnivProbe, Table 1) as an internal control to monitor for successful DNA extraction. This universal (Univ) set was designed to amplify DNA from all eukaryotes, with an annealing temperature similar to the *P. ramorum*-specific (Pram) set to allow multiplex reactions. Primer concentrations were optimized in first and second rounds of PCR to maximize detection rates of the universal fragment while maintaining detection of *P. ramorum* (data not shown). To normalize reactions for quantitative PCR (qPCR), the Univ primer set was further

used to quantify the total DNA in a sample.

Specificity of the assay for *P. ramorum* was tested using both the single-round and nested TaqMan protocols on dilutions of DNA standards, ranging from 0.0015 pg to 1,000 pg, extracted from pure cultures of 18 *Phytophthora* species, including *P. ramorum* (Table 2). These species were chosen to include those most closely related to *P. ramorum*, such as *P. lateralis* and *P. hibernalis* (18,22); those which are frequently co-isolated with *P. ramorum*, such as *P. nemorosa* and *P. pseudosyringae* (11,25,38); and a newly-described species, *P. foliorum* sp. nov., that causes symptoms similar to those of *P. ramorum* on *Rhododendron* sp. (azalea) (C. Blomquist, *personal communication*).

Deoxyribonucleic acids were extracted from lyophilized tissue using a CTAB extraction, modified to include an additional column-based purification step. Lyophilized tissue was pulverized with glass beads in a FastPrep® instrument (Bio101, Carlsbad, CA) for 5 to 30 s at 4,000 rpm. After two repetitions of freezing (on dry ice for 2 min) and thawing (at 75°C for 2 min) in 350 µl CTAB, DNA was purified in phenol:chloroform:isoamyl alcohol (25:24:1), and further cleaned by using the Geneclean® Turbo Nucleic Acid Purification kit (Qbiogene, Irvine, CA) according to the manufacturer's instructions. DNA extracts were eluted in 30 µl ultrapure water (nanopurified, autoclaved, and UV-irradiated), and then were stored at –20°C in 0.1X tris-EDTA buffer. Unless noted otherwise, extracts from pure mycelia were diluted 1:1000 in ultra-pure water prior to amplification, while extracts from plants were diluted 1:100. At least two reagent-only negative controls were included each time extractions were performed to identify any DNA contamination originating from this process. Lettuce leaves were used as an additional negative control for field-collected samples, with at least one asymptomatic lettuce piece for every two environmental samples extracted and then amplified alongside the others.

First-round amplification for the nested protocol using the primer set Phyto1/Phyto4 was performed on 6.25 μl of diluted bulk DNA in each 25.00 μl PCR reaction (0.05M KCl, 0.01M Tris at pH 8.3, 0.1mg/ml gelatin, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.5 μM each of primer Phyto1 and Phyto4, 0.01 μM each of Lt1UnivPrimer, and RtUnivPrimer, 1.25U *Taq* Polymerase [Promega, Madison, WI]). Amplifications were carried out in an iCycler thermalcycler (Bio-Rad, Hercules, CA) under the following conditions: 94°C for 1 min 25 s; then 34 cycles at 93°C for 35 s, 62°C for 55 s, and 72°C for 50 s, adding 5 s at each cycle; a final extension at 72°C for 10 min. Ramp rate: 3.3°C/s heating, 2.0°C/s cooling. Products from the first amplification were diluted 1:500 in ultrapure water before the second amplification.

Each TaqMan reaction, whether performed as the second round of the nested protocol or as a free-standing assay, contained (total volume 15 μl): 1 X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 0.2 μM each primer, 0.2 μM each probe, 5 μl template DNA. PCR was performed in an iCycler IQ thermocycler using the conditions: 1 cycle at 50°C for 10 min, 1 cycle at 95°C for 3 min, 40 cycles at 95°C for 15 sec and 60.5°C for 1 min. Ramp rate: 3.3°C/s heating, 2.0°C/s cooling.

Comparison of diagnostic methods

To compare the sensitivity of a single round of TaqMan amplification with detection using a nested TaqMan protocol, DNA was extracted from *P. ramorum*-inoculated leaves and then assayed by single-round and nested Taqman protocols, as described above, except that only the Pram primer and probe set was used. Both TaqMan protocols were also compared with a nested protocol using the intercalating dye SYBR green for detection (12). Asymptomatic leaves of *Rhododendron macrophyllum* and *Lithocarpus densiflora* collected from a *P. ramorum*-free

site in Oregon were obtained from A. Kanaskie (Oregon Department of Forestry). *R. macrophyllum* and *L. densiflora* were chosen as they are species from which it is relatively easy and difficult, respectively, to isolate *P. ramorum* (*personal observation*). Leaves were inoculated with *P. ramorum* by dipping tip-first in a 1 x10^4 zoospore/ml suspension for 1 min. Leaves were incubated at ambient temperature (10-20°C) in a plastic chamber lined with moist paper towels for 1 week. To speed lesion development, 1 week after inoculation the leaves were floated in a depth of 0.5 cm sterile water for 48 h. Water was then poured off, and the leaves were further incubated for 5 days. Fourteen days after inoculation, the leaves were washed with deionized water and 6mm discs, each containing approximately equal portions lesion and healthy tissue, were sampled using a standard hole punch. To provide a negative control, an equal number of leaves of each species were sham inoculated in sterile deionized water and incubated in different chambers than the inoculated leaves.

Zoospores were produced by taking ten 9 mm diameter agar discs from the margin of a 21-day old colony of isolate Pr102 (ATCC MYA-2440) growing on 10% V8 juice agar and incubating them in 20 ml of sterile deionized water in the dark at 18°C for 3 days. Zoospore release was induced by cold shocking at 4°C for 30 min. After one hour at room temperature, zoospores were counted with an haemocytometer and then appropriately diluted to working concentrations.

For each species inoculated, 30 samples—each consisting of 2 discs—were placed in 2 ml centrifuge tubes and DNA was extracted as previously described. Infection (or lack thereof in uninoculated leaves) was confirmed by plating fifteen discs from inoculated and uninoculated leaves of each plant species onto P_{10}ARP *Phytophthora*-selective agar (per liter: 17 g corn meal

agar, 0.25 g Ampicillin, 0.4 ml of 2.5% Pimaricin, 0.01 g Rifampicin in 1 ml DMSO, 5 ml of 0.5% PCNB in ethanol).

Comparing diagnostic methods using laboratory-inoculated leaves poses a special problem: inoculum levels in such leaves are higher and more uniform than in field-collected tissue. Consequently, DNA extracted from inoculated plants was diluted 1:1,000, 1:10,000, and 1:100,000 with ultrapure water to decrease *P. ramorum* DNA to a concentration low enough to differentiate the sensitivities of the detection methods, and to mimic the range of variability in natural infection conditions (often characterized by DNA that is degraded or in limited amounts). These dilutions were supplemented in equal volume with a 1:100 dilution of extracted DNA of non-inoculated leaves of the same species, which is the usual dilution factor of plant tissue extracts used for diagnostic PCR. This was added to maintain a high level of plant DNA extract while reducing the relative amount of *P. ramorum* DNA. To prevent repeated freezing and thawing, extracts were divided into equal aliquots and stored at –20°C.

Eight replicate samples of each inoculated plant species at each dilution, as well as the non-inoculated controls, and 20 ultrapure water controls were distributed randomly across a 96-well PCR plate for each run. There were 4 replicate plate runs for each PCR technique. A single aliquot of extracted DNA for each treatment was used to perform both single-round TaqMan PCR and the first round of PCR for the nested techniques. The undiluted first-round PCR product was stored at –4°C for 0-20 days before second-round PCR was performed.

Positives for single-round and nested TaqMan PCR were determined using threshold cross times (threshold cycle, or Ct). If the sample's Ct was 40 cycles or less it was scored as positive.This threshold was selected because, at times, non specific fluorescence was detected in negative controls after the 40th Ct. Positives for the nested SYBR protocol were determined using

DNA melt curves and agarose gel electrophoresis. Effects of host, method, and dilution factor were determined with analysis of variance (ANOVA), while differences across methods within dilution factors and host were determined using a Tukey test for multiple comparisons, both using the software JMP v5.01 (SAS Institute, Cary, NC).

Environmental application

In order to verify and compare the efficacy of the above diagnostic methods, tissue from 874 individual plants displaying symptoms putatively attributed to *P. ramorum* was assayed for the pathogen between June 2003 and January 2005. All samples were collected in sealed plastic bags and delivered to the lab, where they were frozen at –80°C prior to nucleic acid extraction and evaluation using the multiplexed, nested TaqMan protocol. These samples originated from 17 California counties, including 13 in which the pathogen had not been isolated at the time of sampling (Tuolumne, Del Norte, Humboldt, Placer, Yuba, El Dorado, Plumas, Butte, Nevada, Sierra, Fresno, Kings, and San Francisco Counties). Thirty-five plant species were tested, including 18 not known to be hosts at the processing time.

To compare relative success of the single-round and nested TaqMan protocols under field conditions, we also assayed a subset of 207 leaf and wood samples with a single round of TaqMan detection.

For all leaf samples, DNA was extracted using the method previously described. Because in our experience this method does not adequately extract or purify DNA from wood tissue, 310 wood/bark samples were processed using a QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA).

Quantitative PCR

In order to quantify the increase in pathogen DNA during early stages of infection, the ratio of pathogen DNA to host DNA was determined in leaf lesions of *Umbellularia californica* individuals, 24, 48, and 72 h after inoculation. Two trials were conducted. For trial 1, leaves were sampled from 9 trees; 3 individuals of low, 3 of intermediate, and 3 of high resistance to *P. ramorum*, as determined by size of lesion size after inoculation in prior experiments (D. Hüberli, *unpublished data*) with 10 leaves sampled per tree. For trial 2, 30 leaves were sampled from each of 3 trees, one each high, medium, or low resistance. Leaves were inoculated by inserting the leaves tip first into 50 ml conical polypropylene tubes. Three hundred µl of a zoospore suspension (2 X 10^4 zoospores/ml) of *P. ramorum* isolate Pr52 (CBS110537, ATCC MYA-2436) were pipeted into the tube. One leaf per tree per treatment was subjected to a sham inoculation using sterile water rather than a zoospore suspension. To reduce premature zoospore encystment, all plastic and glassware in contact with zoospores were acidified prior to use by soaking in 5 M hydrochloric acid for approximately 24 hr and then thoroughly rinsed with deionized water. Tubes with leaves and inoculum were placed in plastic chambers with damp paper towels, and incubated overnight at 18°C. After 18 h, leaves were removed from the tubes, blotted, and incubated on paper towels moistened with sterile water in plastic chambers at 18°C.

Leaves were removed at 24, 48, or 72 h from the time they were first exposed to the zoospore solution. A piece 1 cm in length, encompassing the entire lesion, was cut from the leaf tip, weighed, then frozen and lyophilized for DNA extraction. All DNA extracts were diluted 1000-fold in ultrapure water (the dilution at which most samples' pathogen and total DNA concentrations were in the quantifiable range) before amplifying with a single round of TaqMan PCR. While it is possible to quantify DNA using nested PCR (12,26), to do so the first round of

amplification must be terminated while all samples are in the linear phase; otherwise a pre-amplification step can mask relative differences in initial template concentration. Therefore, a single round of TaqMan amplification was used, despite its lower sensitivity, in order to simplify the process and reduce error. The primers could not be multiplexed reliably (see Results), therefore in order to ensure reporting by both fluorogenic probes, DNA from each sample was amplified in two separate reactions in the same 96-well PCR reaction plate, once using the Pram primer and probe set and once using the Univ set.

Two sets of DNA standards were used in each qPCR run. The first set was composed of a standard series of *P. ramorum* DNA extracted from pure culture of Pr102, quantified by UV spectrometry, and diluted in 0.1X tris-EDTA buffer to a ten-fold series ranging from 0.0001 ng μl^{-1} to 1 ng μl^{-1}. The lowest quantity of *P. ramorum* that can reliably be amplified by a single round of amplification with this method is 0.00001 ng μl^{-1} (or 50 fg per reaction), however, the curve did not always remain linear through this concentration range, meaning that quantification would be less reliable for that portion of the range where linearity was lost (data not shown). To ensure that the standard series used to quantify *P. ramorum* DNA was affected by the same inhibitors as the experimental set, each dilution in the *P. ramorum* series was then spiked with DNA extracted from asymptomatic *U. californica* to a final concentration equal to that expected in the average experimental sample after dilution (trial 1, 47 pg; trial 2, 50 pg). This concentration was determined by quantifying the total DNA in a subset of 60 extractions randomly chosen from the experimental group by UV spectrometry. Three replicate samples of each standard were amplified using the Pram primer/probe set in each PCR run. The iCycler image analysis program (BioRad, Hercules, CA) was used to construct a standard curve by plotting each standard's Ct against the starting concentration. The thresholds that define Ct were

determined automatically by the software program to maximize linearity of the curve. This curve was subsequently used to extrapolate the starting concentration of *P. ramorum* DNA in each experimental sample (Fig. 2).

A second set of standards was used to quantify the total amount of DNA in each sample. This standardization allows for quantities of *P. ramorum* DNA to be expressed as a proportion of the total DNA present, to account for differences in extraction efficiencies and the amount of tissue sampled. Five microliters from each of 60 extractions randomly chosen from the experimental lot were pooled together, and the DNA concentration of this bulked sample was determined by UV spectrometry. The bulk sample was diluted in 0.1X tris-EDTA buffer to create a ten-fold dilution series, ranging from 0.0001 ng μl^{-1} to 10 ng μl^{-1}. Three replicates of each concentration were amplified with the Universal primer/probe set to create a standard curve, as described above, and used to extrapolate the starting concentration of all eukaryotic DNA in each experimental sample (Fig. 2).

Results were calculated by dividing the measured concentration of all DNA by the measured concentration of *P. ramorum* DNA. The resulting ratio was natural log-transformed to correct for a right-skewed distribution. Any data points falling outside the linear range of either standard curve were discarded because they could not be reliably quantified. We used JMP v5.01 (SAS Institute, Cary, NC) to compare ratios across both trials by ANOVA, where effects tested were incubation time, trial, and PCR run. For trial 1 only, we also tested for the effect of individual tree nested within susceptibility category, with tree as a random effect; and for trial 2 alone, we also tested the effect of individual tree as a random effect. We fit the models iteratively by first including all effects and interactions, and subsequently removing nonsignificant effects.

A Bonferroni correction was applied to establish an experiment-wide error rate of $\alpha = 0.05$. For any significant effect, we performed a Tukey test in JMP to compare means among groups.

RESULTS

Phytophthora ramorum was detected by the Pram set using the nested protocol in quantities as low as 15 fg. No other *Phytopthora* species tested was cross-amplified by this method (Table 2). Universal primers did not interfere with detection of *P. ramorum* when they were at low concentrations in both rounds of nested PCR. However, the converse was not true; if *P. ramorum* was detected, in many cases no product was observed from the Univ primer set (Fig. 3).

Comparison of diagnostic methods

Frequency of detection of *P. ramorum* from infected plant substrates differed significantly across plant host species, methods of detection, and quantity of the pathogen DNA (Table 3). Both nested PCR techniques—DNA detection via SYBR green and DNA detection via a TaqMan probe—were equally sensitive. A single round of TaqMan detection was far less sensitive than either nested method; the pathogen was detected only in the most-concentrated stocks by single-round TaqMan PCR (Fig. 4). By all methods, the pathogen was more easily detected in leaves of *R. macrophyllum* than in *L. densiflora*. In *R. macrophyllum*, both nested methods detected the pathogen in 8 of 8 least-diluted samples, in all 4 replicate runs. At this dilution and in this host, the single round of TaqMan was less sensitive, but not significantly so. However, at both lower concentrations, the single-round TaqMan protocol was significantly less sensitive than the nested methods (Tukey's HSD, $P < 0.05$, Fig. 4a). In *L. densiflora*, single-round TaqMan detection was

significantly less sensitive for all three sample dilutions tested; the pathogen was not detected at all in the most-dilute samples (Tukey's HSD, $P < 0.05$, Fig. 4b).

Environmental application

Out of the 874 plant samples from California woodlands tested, *P. ramorum* was detected in 255 by the method here described. This method was used in conjunction with isolation of a culture and completion of Koch's postulates to name the new hosts *Rosa gymnocarpa* (wood rose) (16) and *Smilacina racemosa* (false Solomon's seal, syn. *Maianthemum racemosum*) (15). In the subset of 207 samples tested by both single-round and nested TaqMan methods, *P. ramorum* was detected in 88 of the 207 samples using the nested protocol, but in only 31 using a single round of TaqMan detection.

Quantitative PCR

There was a significant effect of incubation time on the proportion of *P. ramorum* DNA in infected *U. californica* leaves, in both trials (Table 4). In trial 1, *P. ramorum* DNA increased from a mean of 1.1% (N = 30) at 24 hr to a mean of 5.1% (N = 54) at 48 hr and 4.3% (N = 53) at 72 hr. The mean at 24 hr was significantly different (Tukey's HSD, $P < 0.03$) from those at 48 and 72 hr, which were indistinguishable (Fig 5). There was no significant effect of trial, or treatment by trial interaction, nor was there an effect of PCR run. However, in trial 2, there was a significant difference between the mean at 48 hr (2.9%, N = 62), and the mean at 72 hr (4.8%, N = 68). There was no significant difference in the means among resistance categories or among individuals of *U. californica* in trial 1; however, there was a significant effect of individual in trial 2. The tree that had previously been assayed as the most resistant by lesion area had the least

P. ramorum DNA at all times tested, and the least resistant tree had the most (Tukey's HSD, $P < 0.001$). The tree with intermediate resistance was distinguishable from the others at 72 hr, but not at 24 or 48 hr (Tukey's HSD, $P < 0.05$).

DISCUSSION

The real-time PCR method presented here is not only a sensitive and specific method for the diagnosis of *P. ramorum in planta*, but it can also be used to quantify the pathogen DNA present in infected tissue. The nested assay can detect as little as 15 fg of DNA from pure-culture pathogen tissue, and did not cross-react with any of the 18 other *Phytophthora* species tested. These species included the pathogen's closest relatives, as well as those with which it is frequently co-isolated, so the chances of false-positive identification of other species by this method are remote.

The single-round TaqMan assay was significantly less sensitive than both nested techniques in both comparative studies presented here. The nested TaqMan assay, including a first-round amplification step, is as sensitive as an earlier published real-time PCR method using SYBR green for detection (12). TaqMan technology provides a reliable quantification limited only to the desired amplification product (13), while the intercalating dye SYBR green will quantify any double-stranded DNA product, including primer dimers and possibly nonspecific amplicons, and thus necessitates a further step to confirm product identity (41), which can be omitted when using TaqMan chemistry. We did not compare all methods for detection of *P. ramorum* (e.g., 5,7,20,23,33), however, our data reinforce that, even with a multicopy target DNA region, a single round of PCR is not sufficient to detect *P. ramorum* in some substrates, especially when small amounts of the pathogen are present. Martin et al. (23) demonstrated that

the addition of plant DNA extracts to solutions of pure pathogen DNA can dramatically reduce sensitivity of a PCR-based assay for *P. ramorum*. Our data further demonstrate that the *identity* of the plant host matters; an assay that is sufficient for detecting the pathogen in one host may not be acceptable in another. Substrate is a critical issue for pathogen detection, and cannot be ignored when developing or validating techniques.

In addition to laboratory testing, the nested TaqMan method described here proved useful as a tool for detecting *P. ramorum* in field-collected samples. It was used to assay hundreds of samples from throughout California, and, along with the completion of Koch's postulates, helped to confirm the identity of new hosts. The relative success of single-round and nested amplifications in detecting the pathogen was consistent in laboratory-inoculated and field-collected samples. In both cases, sensitivity of the assay was markedly increased with the nested protocol, and detection rates increased more than twofold.

The universal primer and probe set we describe here can be used both as a control for successful extraction, to confirm that DNA is present, and as a control for differences in extraction efficiency or sample amount when quantifying the pathogen DNA present within a host.

The universal primer set did not interfere with frequency of pathogen detection by the *P. ramorum*-specific set, thus, it is possible to multiplex the two primer sets if the goal of amplification is solely detection of *P. ramorum*. However, pathogen detection by the Pram set sometimes results in loss of the Univ signal. Therefore, to normalize samples for qPCR using universal primers, pathogen DNA and total DNA must be quantified in two separate reactions, each with its own primer set.

We used this qPCR approach to document an increase in the quantity of *P. ramorum* DNA within an infected leaf over the course of infection. The significant effect of individual in one trial, though not the other, suggests that with sufficient sample numbers, it may be possible to assay host resistance to *P ramorum* using qPCR within one to three days of inoculation. Lesion expansion rate is one measure by which host resistance to pathogen infection is often assayed (e.g., 3,8,14,24); our technique may be a method to assay resistance at a finer scale.

Furthermore, the ability to detect *P. ramorum* in inoculated leaves 24 hours after exposure to zoospores, despite the absence of significant visible disease symptoms, may be used to design rapid tests aimed at differentiating susceptible and non-susceptible plant species (e.g., interspecific comparisons). Even with relative small sample sizes, it may be possible to detect intraspecific differences among individuals with a larger range of susceptibility.

In conclusion, this study demonstrates that PCR-based diagnostic assays differ significantly in their sensitivity, and that each new assay must be tested on a variety of substrates and at varying concentrations of target DNA before being implemented. The technique presented here provides a method by which *P. ramorum* may be both detected and quantified in plant material, even in the presence of inhibitors and at low concentrations of target DNA. The universal primers and fluorogenic probe may serve as a control to assure that nucleic acid extraction has been successful; alternately, they provide a method by which host DNA may be quantified as a benchmark to compare quantities of pathogen DNA. Thus, qPCR provides a relatively quick method for quantifying pathogen growth *in planta*; in combination with histological studies (e.g., 27), it promises to considerably expand understanding of this economically and ecologically important pathogen and its effects on a broad range of hosts.

ACKNOWLEDGEMENTS

We thank A. Kanaskie for providing asymptomatic *R. macrophyllum* and *L. densiflora* leaves; C. Blomquist, M. Coffey, D. Mitchell, D. Rizzo, and P. Tooley, for providing isolates; D. Rizzo, L. Brown, and K. Waring for providing symptomatic plant samples; and M. Roy for TaqMan advice. We are extremely grateful to H. Belmonte, N. Kordesch, K. Maeda, M. Meshriy, S. Swain, J. Tse, and B. Voss for technical assistance, and E. Simms, S. Bergemann, T. Harnik, D. Hüberli, R. Linzer, K. Peay, and M.A. Smith for helpful comments. This research was funded by grants from the Gordon and Betty Moore Foundation and the USDA Forest Service Pacific Southwest Research Station.

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Table 1. Sequence, fragment size, and situation of primers and fluorogenic probes used for detection, quantification, and normalization of *Phytophthora ramorum* DNA.

Oligonucleotide	Sequence (5'-3')	Fragment size (bp)	Region of rDNA
Phyto1[a]	CATGGCGAGCGCTTGA	687	ITS1,5.8S,ITS2
Phyto4[a]	GAAGCCGCCAACACAAG		
Pram5	TTAGCTTCGGCTGAACAATG		
Pram6	CAGCTACGGTTCACCAGTCA	73	ITS2
Pram7	(6-FAM)ATGCTTTTTCTGCTGTGGCGGTAA(BHQ1a-6FAM)		
Lt1UnivPrimer	TTGGAGGGCAAGTCTGGT		
RtUnivPrimer	CGAGCTTTTTAACTGCAACAA	82	SSU
UnivProbe	(5HEX)CCGCGGTAATTCCAGCTCCAATAG(BHQ1a-5HEX)		

[a]Hayden *et al*. 2004

Table 2. Isolates of *Phytophthora* species used to determine specificity of reaction of the *P. ramorum*-specific primers and fluorogenic probe, using a nested protocol. Isolate numbers on the same line are synonymous. *Phytophthora ramorum* was the only species amplified by the single-round or nested TaqMan assay.

Species	**Isolate no.**[a]	**Host**	**Origin**
Phytophthora boehmeriae	325[PT]	*Boehmeria nivia*	Papua New Guinea
P. cambivora	PDR198513[CB]	*Quercus agrifolia*	California, USA
P. capsici	302[PT]	*Capsicum annuum*	Florida, USA
P. capsici	P141[DR], 3300[GB]	*Lycopersicon esculentum*	
P. cinnamomi	P6379 (A1) [MC]	*Ananas comosus*	Taiwan
P. cinnamomi	P6379(A2) [MC]	*Persea americana*	California
P. cryptogea	IMI 045168	*L. esculentum*	New Zealand
P. erythroseptica	355[PT]	*Solanum tuberosum*	Maine, USA
P. foliorum sp. nov.	9C[CB]	*Ardisia japonica* 'Chirimen'	California, USA
P. foliorum sp. nov.	18[CB]	*Photinia x. fraseri*	California, USA
P. gonapodyides	393[PT], NY353[WW]	*Malus sylvestris*	New York, USA
P. hibernalis	1895[DR], 379[PT], ATCC60352	*Aquilegia vulgaris*	New Zealand
P. hibernalis	1896[DR], 380[PT], ATCC60352	*Citrus sinensus*	Portugal
P. hibernalis	1894[DR], 338[PT], ATCC56353	*C. sinensus*	Australia
P. ilicis	4175a[EH]	*Ilex aquifolium*	Oregon, USA
P. lateralis	PL16[DR]	soil	California, USA
P. lateralis	PL27[DR]	*Taxus brevifolia*	California, USA
P. lateralis	PL33[DR]	*Chamaecyparis lawsoniana*	California, USA
P. megasperma	309[PT], 336[PH]	*Pseudotsuga menziesii*	Washington, USA
P. nemorosa	P16[DR]	*Umbellularia californica*	California, USA
P. nemorosa	P44[DR]	*U. californica*	California, USA
P. nicotianae	P1352[MC], 331[PT]	*Nicotiana tabacum*	North Carolina, USA
P. palmivora	P1-10[DJM]	*Theobroma cacao*	Costa Rica
P. pseudosyringae	P40[DR]	*Q. agrifolia*	California, USA
P. syringae	PDR115773A[CB]	*Rhododendron* sp.	California, USA
P. ramorum	Pr-01[DR], CBS110534	*Q. agrifolia*	California, USA
P. ramorum	Pr-06[DR], ATCC MYA-2435	*Q. agrifolia*	California, USA
P. ramorum	Pr-13[DR]	*Q. agrifolia*	California, USA
P. ramorum	Pr-36[DR], CBS110953	*Q. agrifolia*	California, USA
P. ramorum	Pr-52[DR], CBS110537, ATCC MYA-2436	*Rhododendron sp.*	California, USA

[a]Isolate source: CB, Cheryl Blomquist; GB, Greg Browne; MC, Mike Coffey; PH, Phil Hamm; EH, Everett Hansen;

P. ramorum	Pr-72[DR], CBS110954	*Rhododendron sp.*	California, USA
P. ramorum	Pr-102[DR], ATCC MYA-2949	*Q. agrifolia*	California, USA
P. ramorum	Pr-105[DR]	*Lithocarpus densiflora*	California, USA
P. ramorum	Pr-106[DR], CBS110956	*U. californica*	California, USA
P. ramorum	Pr-108[DR]	*U. californica*	California, USA
P. ramorum	Pr-114[DR]	*U. californica*	California, USA
P. ramorum	Pr-120[DR]	*L. densiflora*	California, USA
P. ramorum	Pr-159[DR], CBS110543	*L. densiflora*	California, USA
P. ramorum	Pr-SDC21.6[DR]	*Sequoia sempervirens*	California, USA
P. ramorum	BBA 12/98[SW], CBS101551	*R. catawbiense* 'Grandiflorum'	Germany
P. ramorum	Phyram1[EM]	*R. catawbiense* 'Grandiflorum'	Mallorca, Spain

DJM, Dave Mitchell; EM, Eduardo Moralejo; PT, Paul Tooley; DR, David Rizzo; SW, Sabine Werres; WW, Wayne Wilcox.

Table 3. Effect tests by ANOVA on detection frequency of *P. ramorum* in four replicate PCR runs. Two plant hosts were tested, as well as three detection methods (nested and non-nested TaqMan PCR, and nested PCR with SYBR Green detection), and at three dilutions of DNA extracted from infected hosts. All dilutions were supplemented to contain standard amounts of plant DNA extracts.

Source	**DF**	**SS**	**MS**	**F Ratio**	**Prob > F**
Host	1	2.217	2.217	60.819	<.0001
Dilution	2	5.077	2.539	69.650	<.0001
Method	2	0.847	0.423	11.617	<.0001
Method*Dilution	4	0.366	0.092	2.513	0.0481
Error	80	2.916	0.036		

Table 4. Effect tests by restricted maximum-likelihood analysis of variance on the quantity of *Phytophthora ramorum* DNA present in infected *Umbellularia californica* leaves[a]

Source	df	SS	F ratio	P>F	Variance Component	Percent of Total Variance
Trials 1 and 2						
Days	2	136.866	26.543	<0.0001	…	…
Trial	1	0.10433	0.0405	0.8407	…	…
Days x trial	2	10.983	2.1299	0.1205	…	…
Error	333	858.521	…	…	…	…
Trial 1						
Days	2	41.045	4.658	0.0342	…	…
Susceptibility	2	30.371	3.447	0.1008	…	…
Days x susceptibility	4	7.263	0.412	0.7965	…	…
Tree[susceptibility] (random)	6	4.885	…	…	0.0581	1.29
Days x tree[susceptibility] (random)	11	3.357	…	…	0.0417	0.925
Error	111	489.032	…	…	4.406	97.785
Trial 2						
Days	2	45.147	27.048	0.0047	…	…
Tree (random)	2	74.966	…	…	0.516	36.689
Days x tree (random)	4	8.506	…	…	0.057	4.026
Error	193	161.087	…	…	0.835	59.284

[a] Days = days incubation after inoculation (1, 2, or 3 days). Susceptibility = high, medium, or low susceptibility of the tree from which leaves were taken, as determined by prior inoculations (D. Hüberli, *personal communication*).

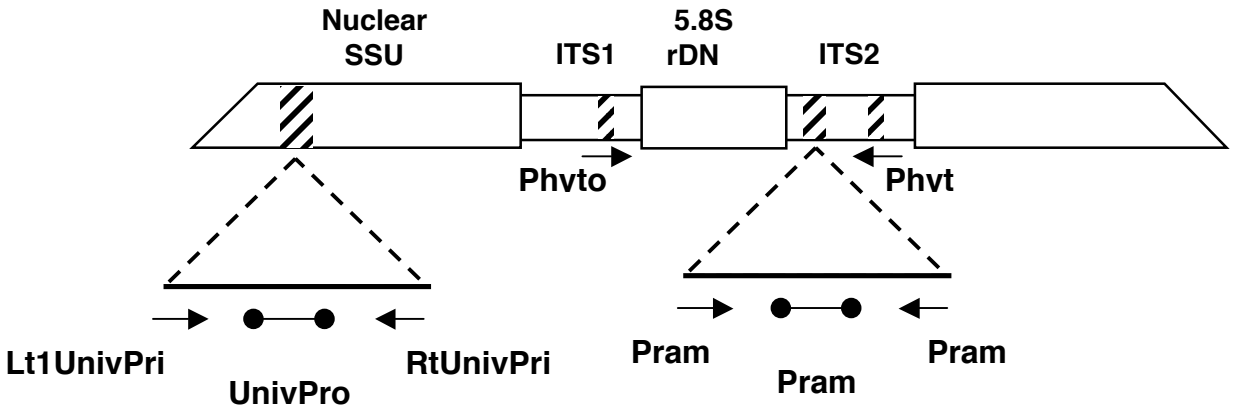

Fig. 1. Relative locations of primers and fluorogenic probes (Table 1) within the nuclear ribosomal DNA region. Hatched regions represent sites of specific binding. Primers are indicated with single-headed arrows, and probes with double-headed bars. ITS, internal transcribed spacer; LSU, large subunit; SSU, small subunit.

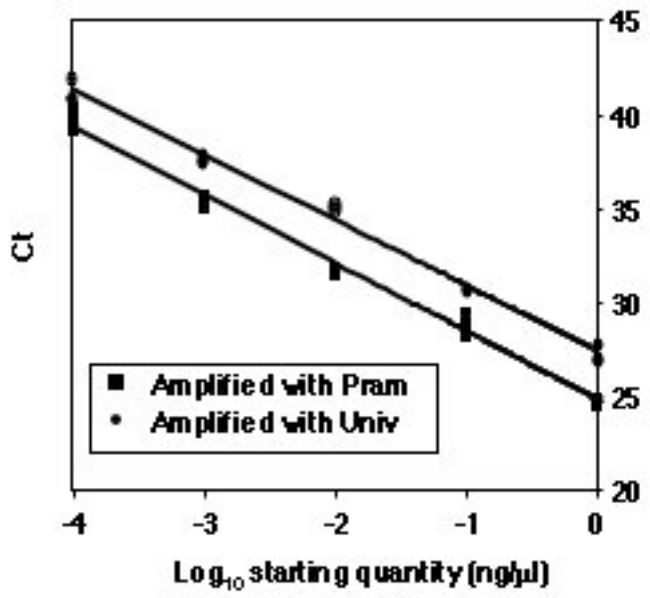

Fig.2. Standard curves for quantifying *Phytophthora ramorum* within infected tissue. With every PCR run, curves were calculated for both *P. ramorum*, using known quantities of *P. ramorum* DNA spiked with extract from uninfected host leaves and amplified with the *P. ramorum*-specific primers and TaqMan probe (squares, $R^2 = 0.996$); and total DNA, using DNA extracts from a subset of the experimental samples, pooled together, quantified by UV-spectrophotometry, and amplified using the universal primers and probe (circles, $R^2 = 0.996$). "Ct" is the threshold cross time, or the amplification cycle at which a sample's fluorescence rises above a threshold value. Quantity of *P. ramorum* in unknown samples was expressed as a proportion of the total DNA present, as extrapolated from the standard curves.

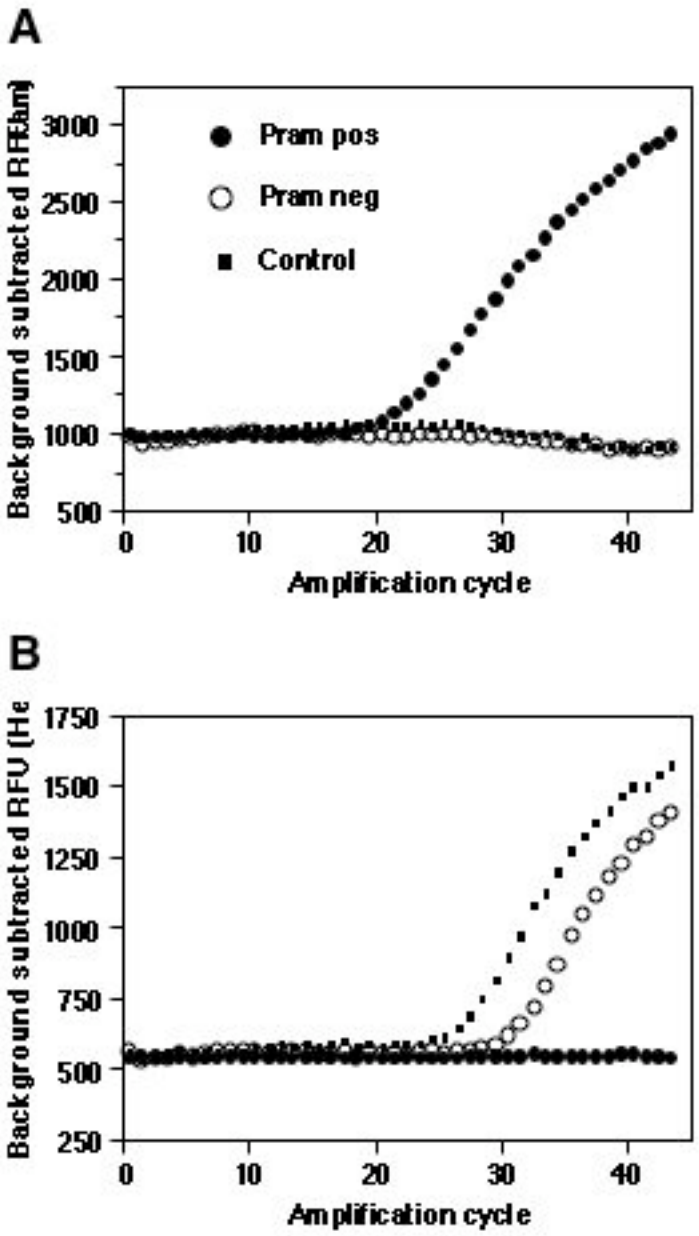

Fig. 3. Interference of UnivProbe reporting by the Pram7 product. Each multiplexed TaqMan PCR reaction generates two amplification curves, one from each fluorophore's excitation and emission filters. RFU is relative fluorescence units. Fig. 3A: Leaf tissue infected with *Phytophthora ramorum* shows fluorescence above a baseline threshold using the Fam-labeled probe Pram7 (closed circles, Pram pos), while neither *P. ramorum*-negative leaf tissue (open circles, Pram neg), nor lettuce leaves extracted as a control for contamination (boxes, control) fluoresce above the baseline. Fig. 3B: Both *P. ramorum*-negative leaf samples show amplification using the Univ probe, labeled with a Hex fluorophore, demonstrating a successful DNA extraction. However, the Univ probe fails to report the *P. ramorum*-positive leaf.

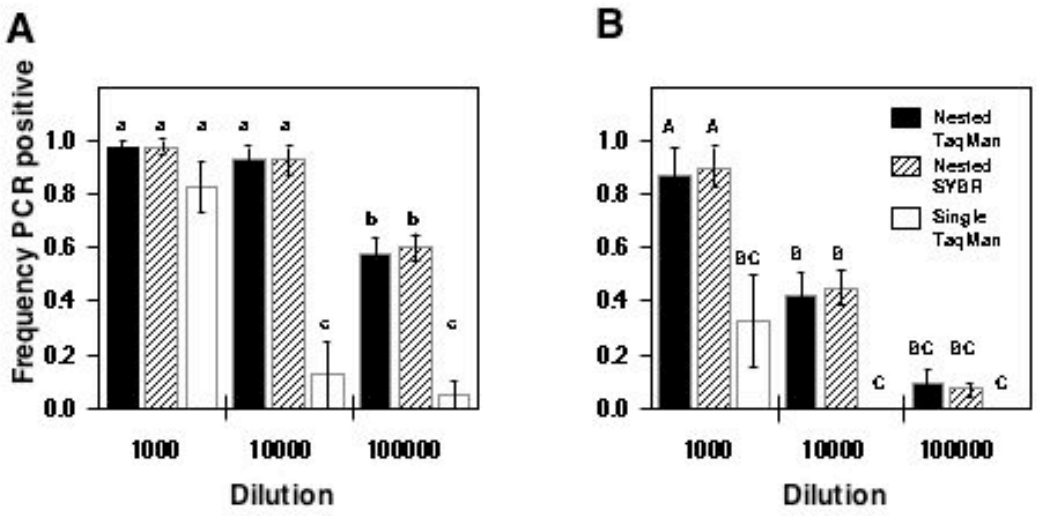

Fig. 4. Relative success in detecting *Phytophthora ramorum* from infected leaves by the nested TaqMan method presented here, a single round of TaqMan amplification without preamplification, and a nested PCR method using SYBR green for detection. Bars connected by the same letter were not significantly different at P = 0.05; data were analyzed within each host, but not across. Fig. 4A: leaves of *Rhododendron macrophyllum*; Fig. 4B: leaves of *Lithocarpus densiflora*. The data here represent mean detection frequencies across four replicate PCR runs; error bars are one standard error of the mean. Dilution factors are the degree to which original DNA extracts of infected leaf tissue were diluted before PCR amplification.

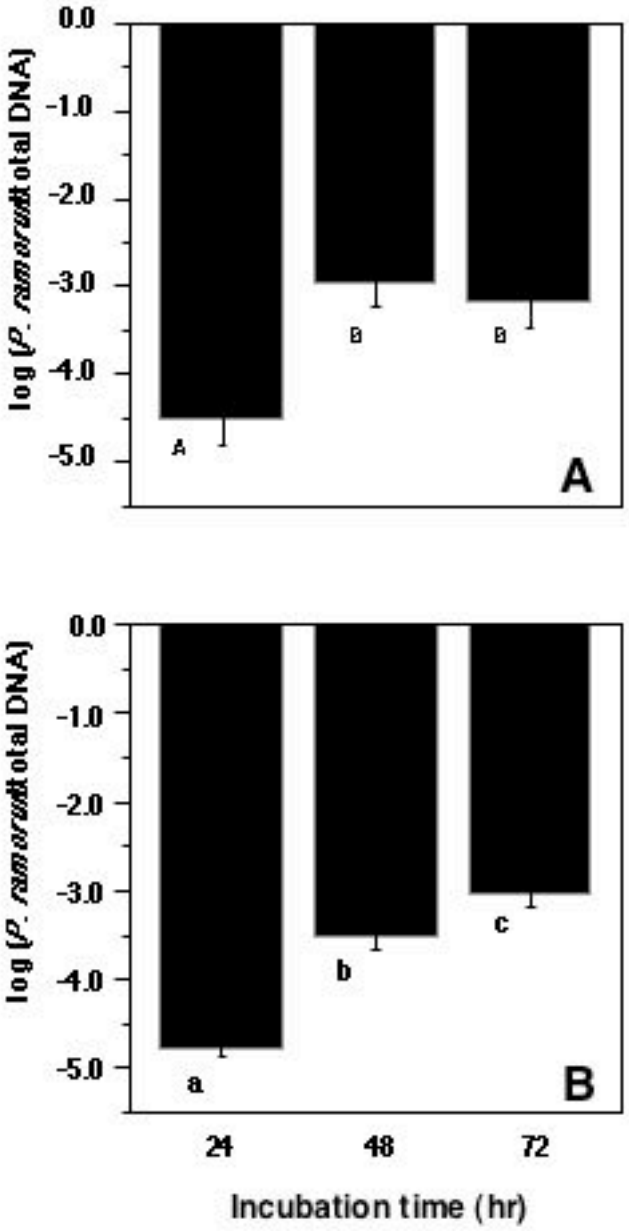

Fig. 5. Quantity of *Phytophthora ramorum* in infected *Umbellularia californica* leaves during the early stages of an infection, represented as a proportion of the total DNA in each sample. Groups significantly different from each other at P = 0.05 within each trial are marked with different letters; error bars represent 1 standard error of the mean. Fig. 5A: trial 1; Fig. 5B: trial 2. There was no significant effect of trial on the mean values.

CHAPTER 3

Can tanoak (*Lithocarpus densiflorus*) adapt to the introduced *Phytophthora ramorum*? An examination of variation in host susceptibility and genetic structure

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ABSTRACT

Although *Lithocarpus densiflorus* is the species most heavily affected by the introduced pathogen *Phytophthora ramorum*, little is known about the origin, range or structuring of its susceptibility to *P. ramorum*. We examined variation in susceptibility to *P. ramorum* using an inoculation assay of detached leaves, from trees at 5 sites. To confirm a correlated response, we assayed foliar and stem inoculations in an additional set of seedlings. The structure of variation in pathogen resistance was compared with variation at nine nuclear microsatellite markers.

We found quantitative variation in susceptibility, with most variation at the individual level (25%), and 12% among populations. Among-population genetic diversity at 9 microsatellite loci was weakly structured (9.5% of variation), with 90.5% of the variation attributable to the intrapopulation level. Among-population genetic diversity was a poor predictor of susceptibility.

We found correlation between leaf lesion susceptibility and lesion expansion in twigs, however trees more susceptible to leaf lesions were not significantly more susceptible to initial infection by *P. ramorum* zoospores. Our findings of limited structure in population genetics and resistance to *P. ramorum* in this highly susceptible host suggest that, while disease progress may be slowed in some populations, all are at risk.

INTRODUCTION

Plant populations and communities can be dramatically altered by disease epidemics (reviewed by Gilbert, 2002; Burdon *et al.,* 2006); in particular, introduced forest pathogens have significantly and irreversibly altered the North American landscape. In some instances, populations of forest trees have been found to have some degree of resistance to invasive pathogens (reviewed by Sniezko, 2006), as in the cases of white pine blister rust (*Cronartium ribicola*) (Kinloch, 1992) and jarrah dieback (*Phytophthora cinnamomi*) (Stuckley & Crane, 1994). In other cases, well exemplified by the chestnut blight (*Cryphonectria parasitica*) epidemic, trees have lacked significant resistance to a newly introduced microbe. In California and southern Oregon, ramorum blight and dieback, also known as sudden oak death (*Phytophthora ramorum*), affects nearly every plant species in the mixed evergreen forest understory and overstory (USDA, 2008). The blight was first reported in one California County in 1995 (Svhira, 1999) and since that time has spread into 14 California counties and in one Oregon. It has moreover been found in nurseries in the U.S., Canada, and Europe (Rizzo *et al.,* 2002; Canadian Food Inspection Agency 2008).

Tanoak trees *(Lithocarpus densiflorus* (Hook. and Arn.) Rehder) are the hosts most susceptible to the ramorum blight pathogen (Davidson *et al.,* 2003; Rizzo & Garbelotto, 2003).

Wildland surveys have found tanoak stands in infested areas to have disease incidence levels ranging between 32% and 90% (Maloney *et al.,* 2005; McPherson *et al.,* 2005). Mortality is generally high throughout the infested region and, although the real impact of the disease on this native tree species is not fully understood, there are indications that the epidemic may devastate tanoak populations. For instance, McPherson and colleagues (2005) found that 24.6% of tanoak trees symptomatic for *P. ramorum* in 2000 in a study site were dead by 2003 and estimated a median survival time for a tree symptomatic in 2000 to be 2.9 – 8.7 years, depending on the severity of symptoms. Over nearly the same period of time, Maloney *et al*. (2005) found average cumulative tanoak mortality to be 9.6% and 15% in infested sites in two different regions, compared to 1.4% and 0.57% in non-infested sites in the same areas. By 2005, the average mortality of tanoak trees was 20.5% in redwood-tanoak forests in the Big Sur, CA ecoregion, with up to 31 standing dead trees per ha (Meentemeyer *et al*. 2008).

Phytophthora ramorum populations have genetic structures suggestive of a recent introduction. Most genetic diversity resides in nursery populations, while only a few closely related, clonally reproducing genotypes are found in US forests (Ivors *et al.,* 2004; Ivors *et al.,* 2006; Prospero *et al.,* 2007). Despite the relative lack of diversity, *P. ramorum* populations show considerable geographic structure (Mascheretti *et al.,* 2008). No host structure has been detected in this generalist pathogen (Rosenzweig & Garbelotto, unpublished data), which is believed to spread primarily from infectious propagules originating from foliar hosts such as California bay laurel (Oregon myrtle, *Umbellularia californica*).

Tanoak trees develop lesions in the leaves and twigs and on the bole; thus, they are not only heavily impacted by the disease, but also may contribute to its spread. Although California bay laurel is the primary source of inoculum in California mixed-evergreen forests, tanoak leaf

and twig lesions do produce spores (Davidson *et al.,* 2008). Bole lesions in the cambium are presumed to be the primary cause of tanoak mortality, but the pathogen has been isolated from xylem tissue and is associated with reductions in sap flow and specific conductivity (Brown & Brasier, 2007; Parke *et al.,* 2007).

Tanoaks grow exclusively on the west coast of the US, ranging from Santa Barbara, California, to southern Oregon in the Coast Ranges, and into some portions of the interior Sierra Nevada and Klamath mountain ranges. The current naturalized range of the pathogen in the US is more limited and lies almost entirely within this area. The geographic mosaic theory of coevolution (Thompson, 1999) provides a useful framework for conceptualizing dynamics in the tanoak-*P. ramorum* system, given its current geographic patchiness. The entire tanoak range may be thought of as a metapopulation, with the infested portions being potential host/pathogen coevolutionary hot spots. Ultimately, whether the host metapopulation as a whole adapts to the pathogen (gains resistance), remains maladapted (more susceptible), or experiences geographically and temporally fluctuating instances of adaptation and maladaptation will depend on genetic variation in host resistance and pathogen virulence, selection mosaics, and gene flow within both host and pathogen metapopulations (e.g., Burdon & Thrall, 1999; Gomulkiewicz *et al.,* 2000; Nuismer *et al.,* 2003).

No tanoak has ever been reported to be qualitatively resistant to *P. ramorum*. The species' fate may depend then, on whether any trees are able to survive, by limiting infection or symptom development, as opposed to resisting infection completely. This type of resistance is known variously as tolerance, quantitative resistance, or reduced susceptibility, with different authors adopting different usages. Usage of the term "resistance" hereafter refers to the extent to which any given host tree limits spread of the pathogen within its tissues (and conversely,

susceptibility is the extent to which pathogen colonization spreads). Quantitative resistance is largely believed to be more durable than qualitative, major gene resistance that is typified by the classic gene-for-gene model (Flor, 1951) and which is more easily overcome through evolution of greater virulence (e.g., Kinloch *et al.,* 2004; reviews in Carson & Carson, 1989; McDonald & Linde, 2002; Sniezko, 2006).

To date, no studies of individual- or population-level variation in resistance in tanoak have been published. Given tanoak's high susceptibility and limited geographic range, such knowledge, combined with understanding of inter-population genetic differentiation and gene flow, is crucial if predictions are to be made about the persistence of the species over the course of the *ramorum*-blight epidemic, with or without human management intervention.

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

MATERIALS AND METHODS

Field survey: Host susceptibility

We set out to determine how resistance to *P. ramorum* was structured within the geographic range of *L. densiflorus*. We sampled five sites within California: the Los Padres National Forest, Monterey County (LP, Los Padres,); the Soquel Demonstration State Forest, Santa Cruz County (SQ, Soquel,); Point Reyes National Sea Shore, Marin County (PR, Point Reyes,); the King Range National Conservation Area, Humboldt County (KR, King Range,); and the University of California Blodgett Forest Research Station, El Dorado County (BL, Blodgett,)

(Fig. 1, Table 1). Climate data for the four weeks prior to collection were extracted from the nearest available weather station within microclimate, except for Soquel precipitation data, which were taken by Brook Kraeger at the Soquel Demonstration State Forest. While both pathogen and host are found further north, in coastal Oregon, the sampled sites nearly span the current range of the pathogen in the United States and are a good representation of the geographic range of *L. densiflorus*. Sites in infested areas were limited to plots that had experienced relatively low mortality from the disease (less than 30%) at the time of sampling and are thus assumed to have been in relatively early stages of the disease.

At each site, 10 trees per transect were tagged at 10 m intervals on each of three 100 m transects. Multiple small branches were sampled from each tree at each site over a 4-day period and stored at 12 °C until collections were complete. Ten replicate leaves per tree were assayed with a detached leaf inoculation, whereby a plug of 10% V8 agar containing mycelia from *P. ramorum* isolate Pr75 was placed on the cut petiole. Inoculations from all the sites were performed on the same day and in random order. Pr75 was originally isolated from *Quercus agrifolia* Née, and was inoculated and re-isolated from *L. densiflorus* prior to this experiment, to ensure that the culture had not lost viability while in storage. Isolate Pr75 is of intermediate pathogenicity on both *Q. agrifolia* and *Umbellularia californica*, as compared to 44 other *P. ramorum* isolates (D. Hüberli, personal communication). The inoculated leaves, plus one sham inoculation per tree, were incubated at 18–20°C in randomly assigned moist chambers for two weeks. At the end of the incubation period, leaves were digitally scanned, leaf and lesion areas were measured using the software program ASSESS (APS, Winnipeg, Canada), and leaf pieces were plated on PARP selective media (Erwin & Ribeiro, 1996) to confirm *P. ramorum* infection.

We examined the distribution of lesion areas within and among sites using a nested ANOVA model with tree nested within population and treating tree and population as random effects and leaf area and moist chamber as fixed effects. Measurements from two chambers were significantly different from the others; these and the replicates they included were excluded from analysis. Variance components were estimated using the restricted maximum likelihood method (REML). Analyses were carried out in JMP v5.0 (SAS Institute, Cary, NC, USA) and in R (R Development Core Team 2009). We calculated the upper bound of broad-sense heritability for lesion area as repeatability, $r = V_{among\ trees} / (V_{among\ trees} + V_{within\ trees})$ (Lynch & Walsh 1998).

Field survey: Host population structure

To determine the genetic diversity and structure of tanoaks at the 5 survey sites, DNA was extracted from mature leaves taken from 191 trees, including most of the trees that were sampled for resistance as well as additional trees at most sites. At the King Range, we were unable to recover DNA from 15 previously sampled trees because of sample contamination during storage. The number of samples per population ranged from 15 in the King Range to 57 at Soquel. We used a simplified CTAB (cetyltrimethyl ammonium bromide) method (Cullings, 1992) for DNA extraction. Eleven microsatellite loci (LD1, LD3, LD5, LD7, LD8, LD10, LD12, LD13, LD14, LD17, LD19; Morris & Dodd, 2006) were amplified by PCR using one fluorescent primer (HEX or FAM). Primers were multiplexed in five groups: i) LD1, LD3, LD7, LD10, LD14; ii) LD5, LD17; iii) LD12, LD13; iv) LD19; v) LD8. The PCR cocktail contained 1X PCR Buffer, 2.0mM MgCl2, 0.2 mM of each dNTP, 250nM of each reverse primer, 250nM of each fluorescently labeled (FAM or HEX) forward primer, 1 unit of Amplitaq Polymerase (Invitrogen, Carlsbad, CA) and approximately 5 ng template DNA in each 20 µl reaction. Primer

concentration for locus LD3 was 400nM. Touchdown PCR cycling conditions for all loci were as follows: one cycle at 95˚C for 10 min followed by 20 cycles of 45 s at 94˚C, 45 s at 58˚C (lowering 0.5˚C each cycle), and 45 s at 72˚C, followed by a final extension step at 72°C for 45 mins. Reactions were performed on a Techne (UK) Flexigene thermocycler. We mixed 0.75 µl of PCR product with 8 µl of formamide and 0.5 µl of 500 LIZ size standard (Applied Biosystems, Foster City, CA) and we electrophoresed this cocktail on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA). We used GENESCAN 3.7 and GENOTYPER 3.7 (Applied Biosystems, Foster City, CA) to analyze the ABI results.

We checked microsatellite data quality using MICRO-CHECKER (Oosterhout *et al.,* 2004) to investigate possible scoring errors and null alleles. Loci LD13 and LD19 had significant values of homozygote excess consistent with null alleles in at least four of the sampled populations. These loci were excluded from our analyses.

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

Leaf lesion progression vs. twig lesion progression and infectivity

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

Logistic regression of quantitative resistance, evaluated as mean leaf lesion area rank per the previous assay, temperature, and their interaction on the presence or absence of infection was used to determine whether "infectivity" was correlated with quantitative resistance. To ascertain whether lesion spread within twigs was correlated with quantitative resistance, we performed ANOVA of the number of *P. ramorum* isolations per twig, indicating lesion spread, excluding zeros (the zeros were the "absent infection" dependent variable, analyzed above), using the same independent variables.

Correlation between neutral genetic and phenotypic variation

We compared neutral genetic structure (F_{ST}) to P_{ST}, the phenotypic approximation of Q_{ST}, which is a measure of population structure of additive genetic variance in quantitative traits (Spitze, 1993). P_{ST} is problematic, in that phenotypic measurements of individuals in natural populations cannot distinguish between environmental and genetic sources of variance. Nonetheless, with sensitivity tests of assumptions of the amount of between- and within-population variance due to additive genetic effects, the relative magnitudes of P_{ST} and F_{ST} may offer insight into the forces driving among-population trait differentiation (Pujol et al. 2008, Saether et al. 2007). We used the Saether et al. (2007) estimation and sensitivity test of $P_{ST} = (g)Var_{population} / [(g)Var_{population} + (2(h^2)Var_{within\ population})]$, where g is the proportion of variance among populations due to additive genetic effects, h^2, narrow-sense heritability, is the proportion of variance among individuals within populations contributed by additive genetic effects, and $Var_{within\ population}$ is the variance among individual trees plus residual variance. P_{ST} was calculated for the entire dataset for $0 \leq g \leq 1$, and $0 \leq h^2 \leq 0.25$. We assumed relatively low values for h^2 because the upper bound for broad-sense heritability, estimated from the repeatability to be 0.29, is necessarily larger than h^2. This sensitivity test indicated $P_{ST} > F_{ST}$ only where $g \geq 0.2$. So, we assumed g and h^2 to be low and equal, and omitted them from further calculations of overall and pairwise P_{ST}, rather than assign them hypothetical values.

Pairwise P_{ST} was compared to F_{ST} and geographic distance between sites using a Mantel test with the ecodist package in R (formula $P_{ST} \sim F_{ST}$ + geographic distance, 10000 iterations).

To understand the relationship between genetic differentiation in tanoak and the observed level of resistance to *P. ramorum*, we used Mantel tests to test two additional null hypotheses.

$H1_0$ is that genetic similarity at the population level is uncorrelated with phenotypic similarity in resistance to *P. ramorum*; $H2_0$ is that there is no correlation between the individual genetic similarity and the individual response to the pathogen.

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

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

RESULTS

Field survey: Host susceptibility

We found considerable variation in the size of lesions developed by individual trees inoculated with *P. ramorum*. While 25% of the variation in lesion area was among individuals, 12% resided among populations (Table 2). Repeatability, the upper bound for broad-sense heritability of lesion area, was 0.28 (SE 0.03).

Lesion areas varied from 6.7 mm^2 to 118.8 mm^2, with individual trees' means ranging from 11.4 mm^2 (SE 0.97 mm^2) to 59.0 mm^2 (SE 10.0 mm^2). Leaf area was significantly associated with lesion area, so leaf area was included in the model, and population resistance rankings are presented as means adjusted for leaf area. The reasoning for not reporting lesions as percent leaf area covered (PLAC) are discussed in the following subsection. Two sites were significantly different from the others after correction for multiple comparisons with Tukey's HSD: Point Reyes trees were more susceptible (adjusted mean lesion area 29.7 mm^2, SE 1.03 mm^2), while Soquel trees were more resistant (adjusted mean lesion area 22.5 mm^2, SE 1.03 mm^2) (Fig. 2).

Field survey: Host population structure

The two point estimates of genetic diversity among tanoak populations, H_e and R_t, ranged from 0.46 (LP) to 0.58 (BL) and 3.14 (SQ) to 3.90 (BL), respectively (Table 3). Bootstrap 95% confidence intervals of allelic richness per population overlapped among most populations, except for comparisons between SQ - BL. (Fig. 3). Within population estimates of the inbreeding

coefficient, F_{IS}, ranged from 0.05 in SQ to 0.13 in BL. However, none of the estimates was significantly different from zero after correction for multiple comparisons.

Private alleles were detected in all populations, ranging from 2 in KR and LP to 10 in BL. Overall population differentiation, F_{ST}, was 0.097 (95% CI = 0.067 – 0.129). Pairwise F_{ST} estimates ranged from 0.057 between PR and KR to 0.128 between LP and KR. All pairwise F_{ST} values were significantly different from zero ($P < 0.05$, after correction for multiple comparisons, Table 4). Consistent with the species estimate of F_{ST}, the partition of genetic diversity by AMOVA attributed 9.5% ($\Phi_{ST} = 0.095$) of genetic variation to population divergence and 90.5% ($\Phi_{IS} = 0.905$) to intrapopulation diversity (Table 5).

Leaf lesion progression vs. twig lesion progression and infectivity

There was a trend for trees whose leaves were least susceptible to lesion spread to be more resistant to initial infection by *P. ramorum* zoospores, but this relationship was statistically significant only at a level of $P < 0.10$ (Table 6). On the other hand, once infected, twigs from more resistant trees developed smaller lesions than those from more susceptible individuals ($P = 0.02$, Table 7).

Correlation between neutral genetic and phenotypic variation

Where there were significant differences in susceptibility between sites, we found phenotypic differentiation between sites to be larger than neutral genetic differentiation (overall $P_{ST} = 0.27$, SE = 0.04, pairwise P_{ST}, Table 4). There was no significant effect of F_{ST} or geographic distance on P_{ST} [Mantel $r = 0.17$, $P(P_{ST} \sim F_{ST}) = 0.42$, $P(P_{ST} \sim$geographic distance$) = 0.58$, $P(F_{ST} \sim$geographic distance$) = 0.77$].

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

DISCUSSION

The entire geographic range of tanoak has been estimated to be environmentally favorable to the establishment of *P. ramorum* (Kelly *et al*., 2007). This estimation, combined with the high susceptibility of the species, suggests that the pathogen could drastically reduce tanoak populations across the entire range of this tree species. The ultimate outcome is likely to depend on variation in host susceptibility, pathogen virulence, and the genetic structure of (meta)populations of both host and pathogen.

We found significant differences among trees in our measure of quantitative resistance, lesion size, here referred to as susceptibility. These differences showed little geographic structure and most variance resided within populations. However, one population was significantly more susceptible than the others and one was less. Likewise, there were small but significant differences between populations in their genetic diversity. As with variation in susceptibility, more genetic variation resides within populations than among populations.

The populations that showed the highest and the lowest susceptibilities in the inoculation study, Point Reyes and Soquel, had similar levels of genetic diversity, and their 95% CI of allelic richness overlapped. The population with the highest genetic diversity, Blodgett, with $R_t = 3.90$

(SE 0.18) and H_e = 0.58 (SE 0.02), showed average susceptibility. Blodgett, the only inland population sampled in this study, was also the most isolated population, due to the gap in the distribution range of this tree species caused by the valleys located between the interior and the coastal mountain ranges of California. Interestingly, although Blodgett is characterized by the largest number of private alleles, its levels of differentiation from other populations (pairwise F_{ST}) were not especially high overall. In general, our findings of moderate levels of genetic diversity and low population genetic structure are consistent with expectations for a woody species with a regionally-dispersed geographic range, outcrossing breeding system, wind-dispersed pollen and limited, animal-ingested seed dispersal (Hamrick *et al.*, 1992) and are in line with recent studies of the true oaks in California (Dodd *et al.,* 2005, Grivet *et al*., 2008, but see Dutech et al. 2005).

Dodd and colleagues are currently undertaking an extensive study of tanoak population genetics (Nettel *et al*., unpublished data). In an earlier study of 4 tanoak stands at the Jackson State Forest at Mendocino, California, they found within-stand genetic variability that was similar to that reported here, as well as clonal spread, with up to 7 stems in a single clone. Most clones, however, were clustered tightly, and the number of ramets decreased with stand age (Nettel *et al*., 2007). No clones were found in the study reported here, indicating that the 10 m spacing between sampled trees was sufficient to avoid resampling the same genets.

We conclude that tanoak's genetic diversity at the population level as estimated from the microsatellite markers is a poor predictor of susceptibility. Differentiation of Further, Mantel tests showed no relationship between genetic distance and similarity in susceptibility. Since microsatellite markers are likely to be selectively neutral, it is not surprising that we were unable to find a link between these markers and susceptibility. Dodd and colleagues (2005) did not find

a relationship between any AFLP markers (whose greater number and location in coding and non-coding regions alike make finding links more likely) or genetic distance between populations and susceptibility to *P. ramorum* in coast live oak, *Quercus agrifolia*. Anacker and colleagues (2007) did find AFLP markers associated with variation in susceptibility in *U. californica*, nevertheless, in the same study, the authors found environment to have an even greater effect. We used a detached leaf assay to gage susceptibility; this assay was correlated with lesion spread within woody tissue, and so its results relate both to pathogen sporulation and to tree fitness. The correlation between assays was confirmed with assays performed two years apart, indicating that relative susceptibility is durable over time. If the possible correlation between rates of spread of the pathogen and rates of infection by zoospores (Table 7, logistic regression, $P = 0.096$) were to be confirmed, independently of whether genetic or environmental in origin, trees defined as less susceptible may be subject to lower levels of infection and also be characterized by slower progression of disease once infected.

Although repeatability values under 50% in our assays indicate that the environment plays a large role in the determination of tanoak susceptibility, we also found that resistance is likely to be heritable to some degree. The detached-leaf assay had a repeatability of 28% (SE 0.03), in line with the single-inoculation values reported by Dodd and colleagues (2008) using a detached-branch *P. ramorum* inoculation of *Q. agrifolia*. Repeatability provides an upper bound estimate for broad-sense heritability and low values such as these indicate that environment contributes more to the variance than does genetics (Lynch & Walsh, 1998). Nonetheless, there was no clear link between climatic factors and susceptibility: the populations with extreme phenotypic values did not have extreme temperatures or rainfall in the weeks prior to collection. The most susceptible population, Point Reyes, did have the lowest average minimum daily

temperature, by 0.6°C, while the least, Soquel, had the highest, by 0.3°C, with a 3.5°C difference between the two.

Drawing conclusions about evolutionary forces on traits from phenotypic observations is problematic, because environmental and genetic variance is confounded (Pujol et al. 2008). Nonetheless, if we conservatively assume $g = h^2$ and that both parameters are smaller than the upper bound for broad-sense heritability (which would certainly make sense, because broad-sense heritability encompasses more sources of variability than does narrow-sense), we find no between population-level differentiation between quantitative and genetic traits, so we cannot reject the null model of no differentiation in phenotype, beyond random variation (Felsenstein 1986, Lande 1992, Spitze 1993). Environmental influences could have masked true variation, but P_{ST} estimated from wild populations is unlikely to be less than the "true" Q_{ST} (Leinonen et al. 2007). While better estimates of genetic influence on susceptibility are required to make firm conclusions, this preliminary investigation finds no indication that populations had previously experienced different degrees of selection on susceptibility.

A common garden study is currently underway to better estimate the narrow-sense heritability of susceptibility in tanoak and a field study is in progress to determine the effect of the susceptibility measured in this study on tree survivorship. While the genetic component of resistance variance is bound to be important from an evolutionary perspective, variation in the realized persistence of host populations will result from genetic and environmental variance in host survivorship and reproduction. Our sampling was too limited to determine whether rare alleles responsible for greater quantitative or qualitative resistance may be present somewhere in the population.

We found a potential for tanoak populations to evolve in response to the *P. ramorum* epidemic, but such an adaptive response faces many obstacles. First, our estimates suggest that heritability of resistance is likely to be quite low. Second, while we found tanoak populations to be significantly genetically structured, differentiation was low, indicating high gene flow. Gene flow may act to swamp adaptation in hot spots by influx from cold spots; this effect will be amplified if the areas of infestation are small relative to the total range of the tree. Conversely, migration may allow marginal populations to persist long enough to adapt by demographically rescuing a declining population and/or increasing genetic diversity, increasing the chances of a novel, more fit gene combination to arise (Gomulkiewicz *et al*., 2000; Lenormand, 2002; Nuismer *et al.,* 2006, Garant *et al*., 2007). Phage studies have supported the positive influence of gene flow on local adaptation in host-parisitoid systems (Forde *et al*., 2004).

Because clones resprout readily from tanoak trees felled by *P. ramorum*, we might expect clonal reproduction to act in much the same manner as a seed bank, which tanoaks lack. Seed banks can maintain genetic diversity in the face of temporally fluctuating selective pressures and also slow evolutionary change (Templeton, 1979). If a "sprout bank" has the same characteristics, the effect would be to extend the life of tanoak stands that would have otherwise been killed outright, but also to dampen the response to selection. Alternatively, clonal reproduction may allow more rapid reproduction by any more resistant genotypes, allowing a speedier response from this relatively slowly maturing species. Survival of sprouts from highly susceptible trees is quite low, anecdotally, so the second alternative may be dominant.

P. ramorum reproduces primarily on *U. californica* (Davidson *et al*., 2003; Davidson *et al*., 2008). Consequently, while adaptation by tanoak may be slow due to the reasons outlined above and the long generation time of forest trees, its evolution of resistance might not elicit a

rapid reciprocal coevolutionary response in the much more quickly reproducing pathogen, which might otherwise be expected for a specialist. In this respect, we would predict that any evolution of resistance to *P. ramorum* in tanoak would be most durable where tanoaks and *U. californica* coexist.

Tanoak is not valued for timber, however the trees are an important food source for wildlife (Barrett *et al.*, 2006), are ectomycorrhizal hosts that help maintain the redwood forest ecotype in which they are codominant (Massicotte *et al.*, 1999; Bergemann & Garbelotto, 2006), and are culturally important to Pacific Coast Native American groups (Meyers *et al.*, 2007). Consequently, the species' ecological and evolutionary trajectory in the face of this introduced disease has broad-reaching implications. The significant differences we found in susceptibility among populations, whether environmental or genetic in origin, provide an important tool for predicting the pace of the epidemic at different sites. We expect the disease to move much faster and have a heavier toll on tanoaks at Point Reyes, the most susceptible site we surveyed, and where the pathogen has only recently been observed, than at Soquel, the least susceptible, where the pathogen has persisted at low levels since at least 2001. Anecdotally, this seems to be the case; monitoring will confirm whether our predictions hold.

The limited genetic and phenotypic structure we found suggest that while disease progress may be slowed in some tanoak populations, all are at risk. Ultimately, disease caused by *P. ramorum* is likely to take a heavy toll on the species. Human intervention, including the development of resistance breeding programs, may be required to ensure long-term survival of this species.

ACKNOWLEDGEMENTS

We thank the members of the Dodd and Garbelotto labs for generous assistance, D. Rizzo for providing isolate Pr75, R. Linzer, K. Peay, and E. Simms for helpful comments, and K. Koy for cartography. The land managers at all 5 sites were immensely helpful, as was Brook Kraeger for providing climatic data. We are grateful for funding provided by the Moore Foundation and the USDA Forest Service PSW field station.

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

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

Table 2. ANOVA (restricted maximum likelihood) of resistance across survey locations; response is lesion area (mm^2). Significance of random factors was tested with model reduction in R.

Source	d.f.	Sum of Squares	F Ratio/ L Ratio	Prob > F
Population	4	2.86	17.94	< 0.0001
Tree(Population)	152	34.40	215.13	< 0.0001
ln(Leaf Area)	1	48.10	714.11	<0.0001
Chamber	37	4.49	1.80	0.0025

	Variance Component	Std Error	95% Lower	95% Upper	% of total
Population	0.012	0.009	0.004	0.139	11.55
Tree(Population)	0.027	0.009	0.020	0.038	25.20
Residual	0.067	0.007			
Total	0.106				

Table 3. Descriptive statistics for sampled populations of tanoak using 9 microsatellite loci: Number of trees sampled (n), Nei's 1987 gene diversity (H_e), allelic richness (R_t), and inbreeding coefficient (F_{IS}). Numbers in parenthesis correspond to the standard error.

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

Table 4. Pairwise estimates of F_{ST} and P_{ST} for the studied tanoak populations. All values of F_{ST} were significantly different from 0 ($P < 0.05$) after correction for multiple comparisons. There was no correlation between F_{ST} and P_{ST} by Mantel tests.

	LP	**SQ**	**PR**	**KR**
F_{ST}				
SQ	0.061			
PR	0.127	0.083		
KR	0.128	0.1	0.057	
BL	0.12	0.1	0.093	0.058
P_{ST}				
SQ	0.054			
PR	0.0811	0.183		
KR	0.005	0.037	0.101	
BL	0	0.064	0.049	0

Table 5. Analysis of molecular variance (AMOVA) of the sampled tanoak populations based on microsatellite markers.

	d.f.	Variance component	% of total variance	P value
Among populations	4	0.23	9.55	<0.001
Within populations	395	2.19	90.45	<0.001

Table 6. Likelihood ratio effect tests for logistic regression of the presence/absence in twigs of *Lithocarpus densiflorus* of *Phytophthora ramorum* after inoculation with zoospores. "Susceptibility" is the ranking of the tree based on the detached leaf inoculation, where 1 was least susceptible.

Source	Nparm	d.f.	L-R ChiSquare	Prob>ChiSq
susceptibility	11	11	17.43	0.0958
incubation temperature	1	1	0	1
temperature* susceptibility	11	11	10.28	0.5056

Table 7. ANOVA of lesion spread within *Lithocarpus densiflorus* twigs, as measured by the number of positive isolations of *Phytophthora ramorum* after zoospore inoculation. "Resistance" is the tree's resistance ranking based on mean lesion area in a detached leaf inoculation.

Source	DF	Sum of Squares	F Ratio	Prob > F
susceptibility	11	27.56	2.04	0.0264
incubation temperature	1	1.80	1.46	0.2278
temperature*susceptibility	11	9.212	0.68	0.7562

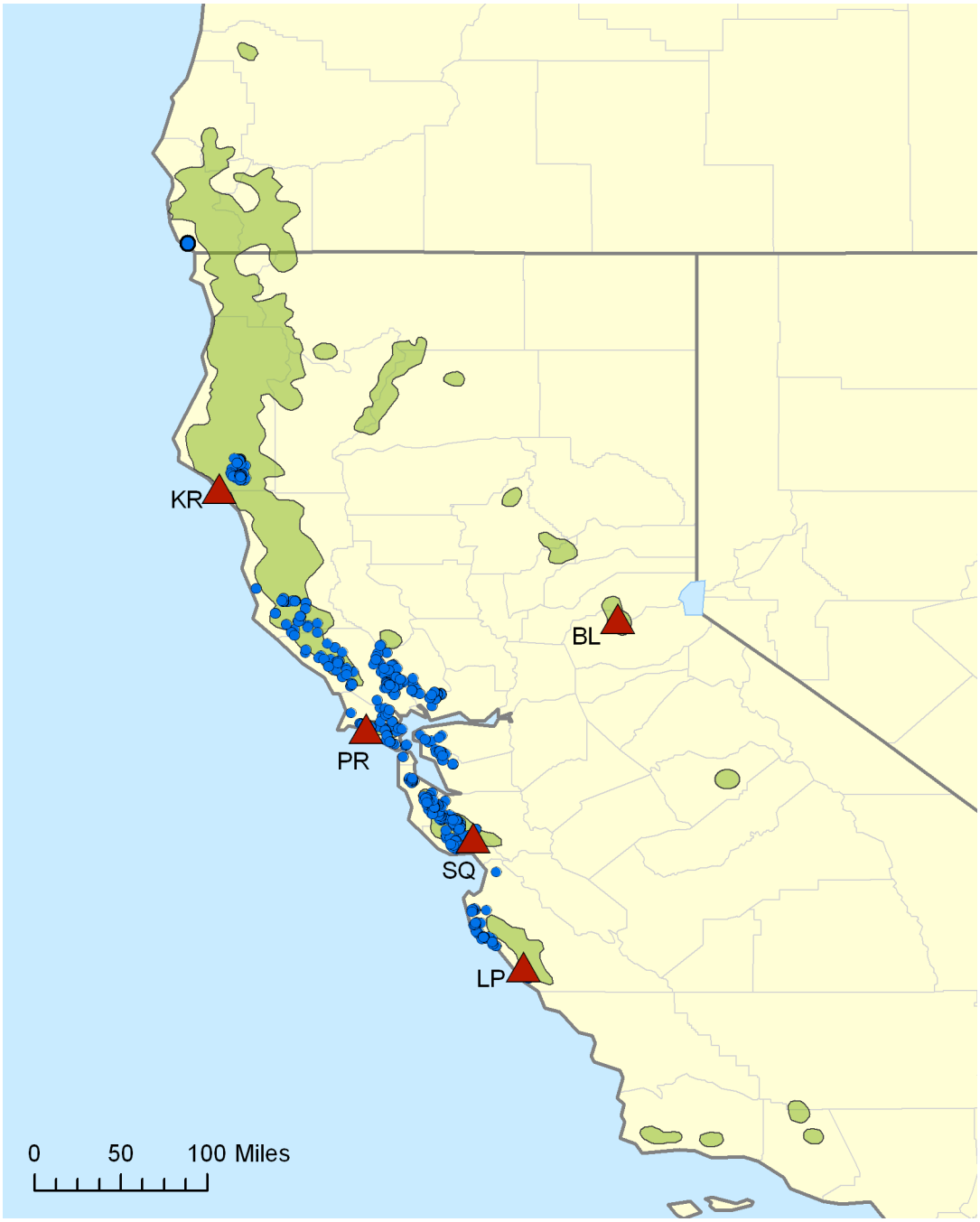


Fig. 1. Location of study populations. Study sites (triangles) are shown in relation to the geographic range of *Lithocarpus densiflorus* (shaded areas, U.S. Department of the Interior 2006), and confirmed findings of *Phytophthora ramorum* (circles; California: Kelly & Tuxin, 2003; Kelly *et al*., 2004; Geospatial Innovation Facility, 2008; Oregon: Oregon Department of Agriculture, 2008). The states shown are Oregon to the North and California to the South.

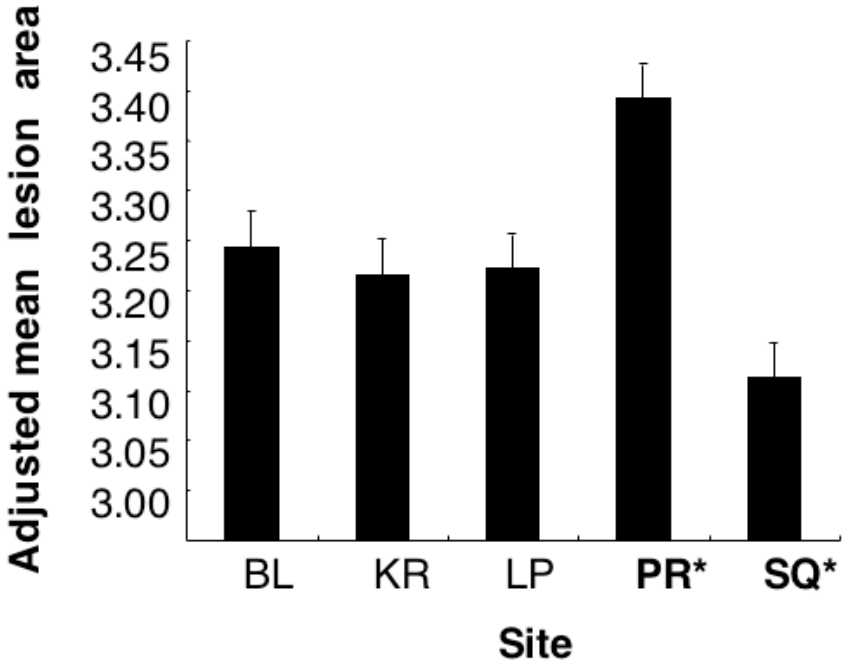


Fig. 2. Mean *Phytophthora ramorum* leaf lesion area of *Lithocarpus densiflorus* at each of five sites, adjusted for leaf area. Data were natural log transformed prior to analysis. Sites with estimates statistically significantly different from the others at $P < 0.05$ are in bold type, and marked with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

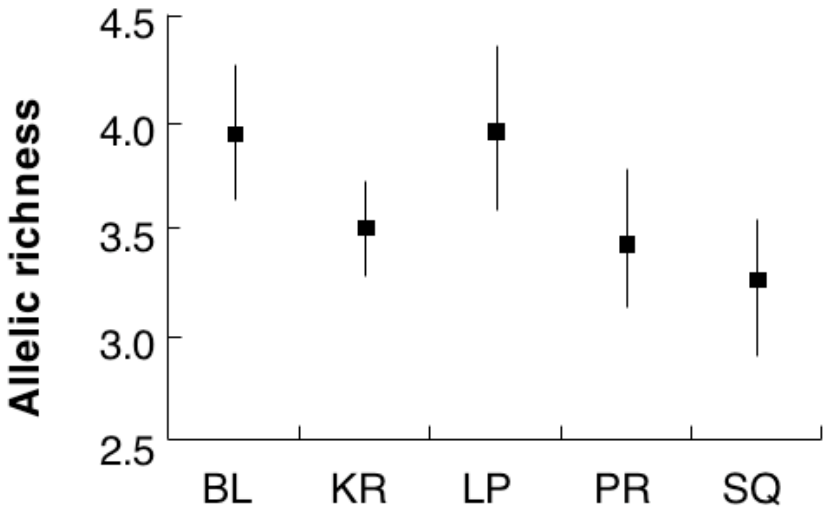
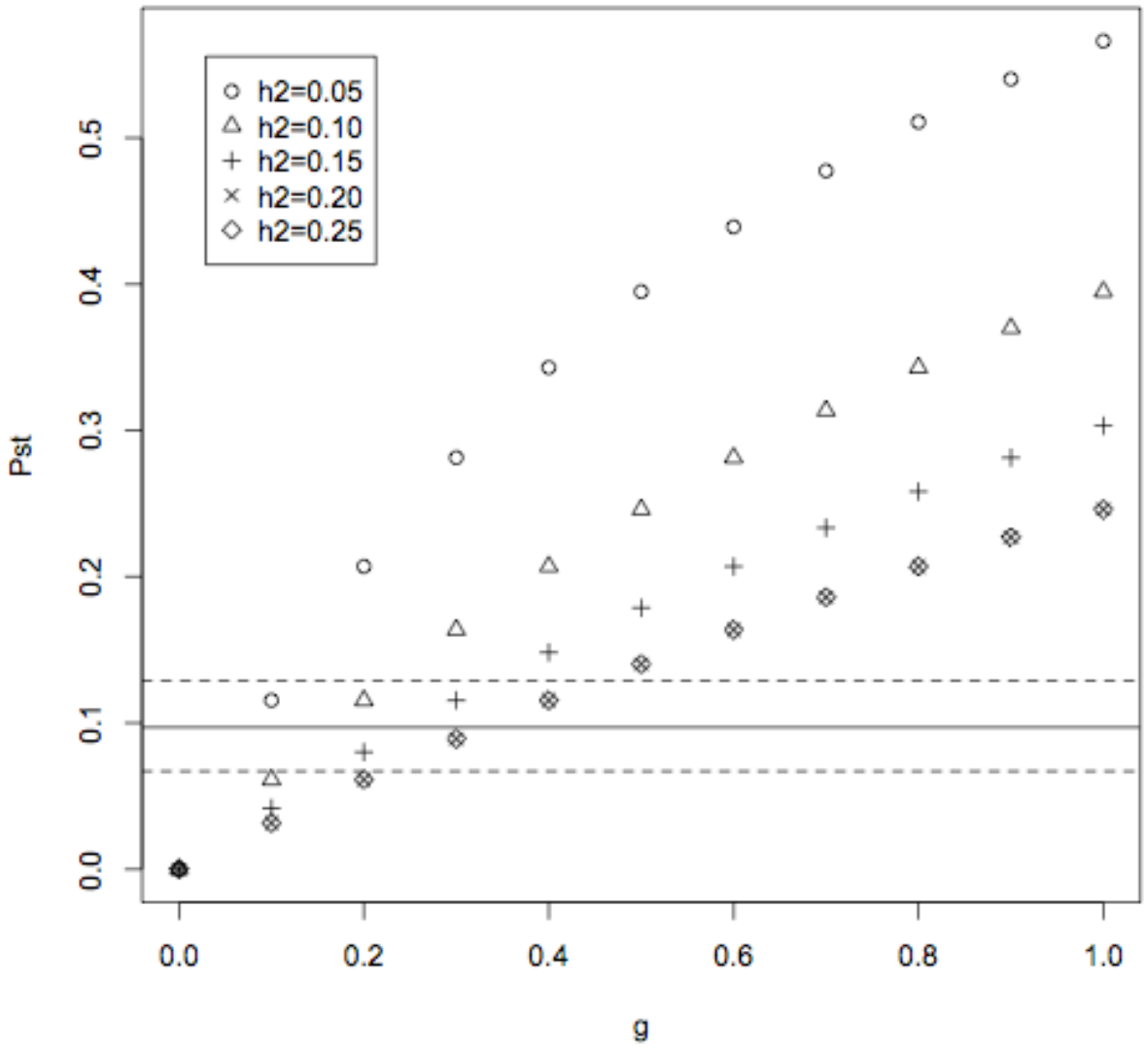


Fig. 3. Plot of tanoak's average allelic richness per population estimated from 9 nuclear microsatellite loci using the rarefaction method. Error bars correspond to the 95% confidence interval estimated from 100 resampled populations.

Appendix A



Appendix A. Sensitivity plot for estimates of P_{ST} of lesion area across all 5 study sites, with different values of narrow-sense heritability, h^2, and the among-population proportion of genetic variance, g. The solid dashed line is the estimate of F_{ST} based on neutral markers, with confidence intervals in dashed lines. When g is low and h^2 approaches its upper bound based on repeatability estimates, P_{ST} and F_{ST} converge.

CHAPTER 4

First studies of heritable resistance to *Phytophthora ramorum* in a tanoak common garden population

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INTRODUCTION

Disease epidemics can inexorably alter forest communities and populations. Introductions of pathogens to naïve hosts through trade or travel seem to have especially dramatic effects; *Chryphonectria parasitica* has upended the forest structure of US Eastern Hardwood forests, reducing the once dominant chestnut to an understory shrub (Anagnostakis 1987), *Phytophthora cinnamomi* has left graveyards of Australian jarrah forests (Weste 2003), and *Ophiostoma ulmi* and *O. novo-ulmi* have traveled across Europe and North America in waves, killing mature elm trees as they go (Brasier 2008). Conversely, introduced pathogens may have mundane effects, so much so that they escape notice (Linzer et al. 2009), and native pathogens can decimate even those plant communities with which they have long been associated with (Hansen & Goheen 2000, Holah et al. 1997). But the mass host mortality seen in epidemics like the single-host chestnut blight or the landscape-wide jarrah dieback seems to be a hallmark of recent introductions (Burdon 1987, Parker & Gilbert 2004), or even the inevitable result of a pathogen

making an occasional sudden jump to a newly available, naïve host population (André & Day 2005).

Even highly susceptible hosts subject to novel disease epidemics may not be completely without resources to resist a new disease, however. Rare individuals may possess major genes resistant to other pathogens and which exhibit a correlated response to the new pathogen. Alternatively, populations might exhibit low levels of more or less nonspecific resistance that limit the extent or severity of infection (Parker & Gilbert 2004, Sniezko 2006). Pathogen resistance is not necessarily an all-or-nothing trait; while the classic R-gene-resistance invokes a highly visible hypersensitive response (HR) that walls off infection, other forms of resistance are measured on a quantitative scale and may not be immediately evident. Western white pine (*Pinus monticola*) and sugar pine (*P. lambertiana*), for example, have both been found to have both low frequencies of major genes for resistance to *Cronartium ribicola*. Both hosts also exhibit forms of "partial resistance," manifested as a reduction in disease incidence and severity (e.g., Kinloch & Byler 1981, Hoff 1986). Long-term field studies of trees bred for these traits have shown that, while major-gene-resistance provided earlier gains in family survival, they could be overcome by a virulent strain of pathogen. In contrast, partial resistance offered some protection against pathogen evolution; some trees with partial resistance maintained increased survival over the whole of the 30-year field trial (Kinloch et al. 2008).

California forests are in the early stages of an epidemic of *Phytophthora ramorum*. The pathogen was first observed in the mid-1990s on dying *Quercus* and tanoak [*Lithocarpus densiflorus* Hook. & Arn., proposed name *Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S. Oh, comb. nov.] trees in Marin County; since that time *P. ramorum* has been found to infect nearly every plant species in the mixed-evergreen understory (Rizzo & Garbelotto

2003). Tanoaks, however, are the most susceptible host and, since the pathogen's first reports, they have been killed in the hundreds of thousands (Meentemeyer et al. 2008). As early as 2002, over 60% mortality was observed in tanoak stands in Sonoma County, CA (Maloney et al. 2005), while McPherson (2005) reported plots near the epicenter of the infestation with upwards of 60-90% of the tanoaks either dead or symptomatic in 2003. Tanoaks' geographic range is restricted to a portion of the California Floristic Province of the western United States (Hickman 1993), and while *P. ramorum* is established in only a subset of that range, the infested area expands yearly (Geospatial Innovation Facility 2010). An understanding of resistance to *P. ramorum* in tanoaks, even at low levels, will be essential for modeling disease dynamics, predicting the evolutionary ecology of the host-pathogen system, and deciding how to mange forests in the face of the potential loss of a key species.

In Chapter 2 of this volume, my colleagues and I reported a survey of detached-leaf susceptibility in adult tanoak trees. Importantly, we found statistically significant variation in susceptibility among individuals, and to a lesser extent among populations. Population structure in tanoak susceptibility echoed variation in susceptibility to *P. ramorum* in clonal cuttings of coast live oak (*Quercus agrifolia*), reported by Dodd et al. (2005), as well as the structure of neutral genetic markers in both tree species.

Because our initial study focused on tissue taken from adult trees in wild populations, two important questions were not answerable: First, how much of the phenotypic variation is heritable – i.e., could it be passed from parent to offspring? Second, are the differences in susceptibility that we observed biologically relevant – that is, can they to contribute to fitness differences under natural conditions?

A quantitative genetics approach is ideal to address the first question. Quantitative genetics is the study of traits which vary continuously and are underlain by allelic variation at numerous loci, like human height or a plant's total leaf area, in contrast to qualitative or either/or traits, such as seed color in peas, which is largely determined by allelic variation at one locus. Quantitative genetic phenotypes are idealized as stemming from a combination of environmental effects and the cumulative action of many genes of small effect, which are all independently distributed and inherited (Lynch & Walsh 1998). These assumptions likely hold for the continuous pattern of variation we observed in leaf susceptibility to *P. ramorum*; indeed, to a much greater extent than plant pathologists in agricultural systems, foresters have long used quantitative genetics for breeding resistant trees (Carson & Carson 1989), and there is a rich literature using a quantitative genetics approach to study plant-pathogen interactions in natural systems (e.g., Fornoni 2004, Frank 1994, Simms & Rausher 1989, Van Tienderen 1991).

The central parameter for understanding, predicting, or manipulating the evolution of quantitative traits is additive genetic variance, the component of phenotypic variance that is passed predictably from parent to offspring, as opposed to maternal effect, dominance interactions, and so on. The proportion of additive genetic variance to total variance, narrow-sense heritability, h^2, can be deduced from resemblances among relatives when the environmental variance is known or held constant (Falconer & Mackay 1996, Lynch & Walsh 1998).

A common garden design is often used to distinguish the differing contributions of genetic and environmental factors in plants: seeds or transplants are collected from various sites, but are reared together in an environment common to none of them (Silvertown & Lovett Doust 1993). The aim is to prevent a genotype by environment covariance: no genotype will be any

more likely to occur in a particular environment than any other, so the effects of the two will not be confounded. Clausen, Keck & Heisen famously (1948) determined a genetic component to morphological differences in *Achillea lanulosa* along an altitudinal transect using such a study.

Heritability is not a constant, however, but a function of genotypic and environmental variance (Feldman 1992). It is necessarily context-specific, and trait heritability in experimental populations is often different from that in the wild (Ritland 2000). Furthermore, unless multiple sites are used, genotype by environment interactions will be missed (Lynch & Walsh 1998, Silvertown & Lovett Doust 1993). Multiple garden sites or reciprocal transplant studies are often introduced to discern such interactions, however even simple reciprocal designs may not be fully capable of differentiating all host-pathogen-environment interactions (Nuismer & Gandon 2008).

A different sort of common garden may also be used to answer the second question. Field trials, in which trees with different genotypes or observed phenotypes are planted in a disease-infested area, are commonly used to evaluate putatively disease-resistant forest trees (Kinloch 2008, Isik 2008). These trials, also known as disease gardens, test whether resistance pbserved in laboratory assays predicts survival under natural conditions (Carson & Carson 1989). Moreover, pathogen resistance occur via many different traits, which may not be correlated with each other. Field trials provide an additional tool with which to judge under which conditions and importantly, in the presence of which inoculum sources, different forms of resistance are useful. They might also identify genotypes not seen in a greenhouse setting (Sniezko 1996).

The characterization of and selection for traits such as pathogen resistance has long been an aim of classic quantitative genetics and methods for using resemblances among relatives to partition additive genetic variation from other sources of variation are well established (Carson & Carson 1989, Falconer & Mackay 1996, Simms & Rausher 1992). However, implementation

effectiveness depends on important knowledge about the population structure and breeding system of a species. Tanoaks present a particular challenge in that, prior to the *P. ramorum* epidemic, they were not economically valued nor commercially propagated in large numbers. Until Nettel's and Dodd's study of tanoak population genetics, a part of which arose from the USFS collaborative project described here, very little information was available about tanoak phylogeography, breeding system, or population structure, all of which would be useful in designing sampling plans and management strategies.

To address this gap, the USDA-Forest Service Pacific Southwest Research Station developed a multi-year collaborative project to develop the infrastructure needed for research on the genetics of susceptibility to *P. ramorum* in tanoak. We report here on the following 3 aims of the project:

1) Establish a common garden of open-pollinated seed. Propagating families in a common environment is the first step towards the calculation of genetic components of quantitative traits and will provide a resource on which other research may be based. Among the research projects supported by the common garden, but not discussed here, will be a provenance study (led by J. Wright, USDA-Forest Service) and a detailed population genetics analysis (led by R. Dodd, UC Berkeley; partially reported in Nettel et al. 2009).

2) Use laboratory inoculation assays to better characterize susceptibility to *P. ramorum* in tanoak and to estimate heritability. In Chapter 3, we reported limited geographic structure and potentially low heritability of lesion development in tanoak leaves from mature trees after inoculation. However, differing source populations and unknown family backgrounds precluded

precise estimates of genetic and environmental effects. Individuals with known family relationships and a common environment may be used to more precisely partition phenotypic variance.

3) Establish a field trial in an infested forest site, to validate and expand on laboratory assays with a study of natural infection dynamics. While, with previous inoculation assays, we did reveal significant variation among individuals, we can say neither if those differences play a role in disease dynamics under natural conditions nor whether the differences will be observed under any other setting. A field trial will allow us to evaluate results from laboratory assays, and better understand of natural disease processes.

METHODS

Establishment of a Common Garden

Since 2006, over 13,000 *N. densiflorus* acorns from 10 unique sites have been collected and planted in pots together in a shadehouse garden in Berkeley, CA, hereafter referred to as simply the common garden (Table 1). Because of the lack of background knowledge on tanoak population genetics at the time the garden was begun, the first collection was intended to survey as much variation as possible. Collection sites were chosen to range from the southern to northern regions of the tree's geographic range, and included one Sierra Nevada population.

Subsequent collections were designed to fill in gaps or target likely resistant populations or individuals, based on resistance assays (see below), or on long-time survival in infested areas. In 2006, only one Sierra Nevada family (BL) had sufficient seed germination to include it in the resistance assays described below, so in 2007, seed was collected from that population alone. In

2008, additional collections were undertaken in the northern part of the range, where there was evidence for admixture of chloroplast haplotypes (Dodd et al. 2009, Nettel et al. 2009), as well as from populations in which mature trees had previously shown differential resistance (this volume, Ch 3).

Open-pollinated seed were collected directly from parent trees between August and November of each year by a combination of collaborators, volunteers, and the authors (Table 1). The acorns were stored in sealed plastic bags at 4° - 6°C until planting. Within each year, all seeds were planted within a 5-day period, starting sometime between the first week of December and the second week of January following their collection. Seed were sown in 6.4 cm x 25 cm conical pots in UC soil mix in a shadehouse in Berkeley, CA, and benches were covered with wire netting for a minimum of 6 months to prevent seed predation.

Seedlings were fertilized yearly with 19-6-12 N-P-K slow-release fertilizer. The seedlings that were assayed by detached-leaves were retained for future use. After two years, seedlings to be kept were transplanted into 10 cm x 36 cm pots in UC soil mix.

Inoculation Assays, 2006 Cohort

After at least one year's growth, seedlings were assayed for resistance to *P. ramorum*. In the first year, seedling susceptibility was tested by a detached leaf inoculation the first year, and both a leaf and an intact seedling tip inoculation the second year, for the BL seedlings alone.

A detached leaf assays was preferred for the initial inoculations because it allowed for replication within individuals, and was less destructive than seedling tip inoculations. A seedling will tolerate losing a few leaves, but until clonal propagation of tanoaks may be developed, it is not possible to replicate seedling inoculations on individuals. Moreover, infected individuals

must be discarded after the assay, so results of seedling inoculations may only identify promising parents, rather than seedlings to keep for a second round of selection.

Leaves were inoculated as described in Chapter 3, using mycelial plugs of *P. ramorum* isolate Pr52 (CBS110537; ATCC MYA-2436) in 10% V-8 juice agar. Seedlings from the 2006 collection were tested in February 2008, 13 months after planting. 71 families from 5 sites were represented. Up to four mature leaves each from between 10 and 15 seedlings per family as available, were inoculated and incubated in moist chambers. Because of the large number of leaves, inoculations were performed in random order over 4 days. Each replicate leaf per seedling was incubated in a different chamber, to avoid confounding chamber with individual effects. After 14 days' incubation, the leaves were digitally scanned into image files, and then analyzed using Assess image analysis software (APS, Winnipeg, Canada). Infection was confirmed by isolation by culture on PARP selective media (Erwin & Ribeiro 1996).

Inoculation Assays, 2007 Cohort

The 2007 acorn collection included seedlings only from the BL site. Twenty-three BL families were subjected to detached leaf inoculations in December 2008. Twelve months post-planting, up to four mature leaves were tested from each of 13 – 21 seedlings per family. Data were analyzed as described above. Eleven of the 1731 observations were excluded for illegibility or, in one case, inoculation failure.

Because removing leaves for the detached leaf inoculations entirely defoliated smaller seedlings, intact seedling inoculations were performed on a separate set of 359 seedlings from twenty families that still had seedlings left after the leaf inoculations. Six to 25 seedlings per family were inoculated inoculated with 100ul of a zoospore suspension isolate Pr52 at a

concentration of ~2 x 10^4 spores per mL, dropped into a wax cup (Parafilm®) surrounding a wound produced by removing the three terminal leaves on each tree. Hansen et al. (2005) inoculated seedlings by dipping plants in zoospore solution; our modification allowed for a precise measurement of lesion development. Seedlings were maintained in a greenhouse facility with ambient light and temperatures between 12°C and 21°C.

Lesion development and survival was monitored for 10 months, with the last measurements recorded during October 2009. In this experiment, 10 adjacent seedlings had a zero infection rate and so were presumed to have been skipped during inoculation and therefore excluded from analysis.

Lesion length, measured as a proportion of the stem length, could not be transformed to fit the assumptions of normality. Because the majority of the observations (199/339) occurred at 1 or 0, lesion length divided by seedling length was modeled as following a quasibinomial distribution, with the same effects as for survival.

Each leaf and seedling inoculation mimics natural infection symptoms, but in different ways (Hansen 2005). Inoculated leaves develop a lesion that travels linearly up the midrib, while inoculated seedlings develop a stem lesion that frequently develops into dieback.

Analysis of Leaf Inoculation Assays

Lesions grew linearly along the leaf midrib, so susceptibility was modeled as a log transformation of lesion length relative to the leaf length, the maximum possible lesion growth. Variances were equal by Bartlett's test, so the data were analyzed with a mixed-model analysis of variance in JMP v. 5.01 (SAS Institute, Cary, NC, USA), as

$$y_{ijklmn} = \mu + C_i + S_j + P_{k(j)} + T_{l(k,j)} + E_{ijklm}$$

where y_{ijklmn} is the dependent variable, μ is the grand mean, C is the i^{th} incubation chamber, S_j is the j^{th} source site, $P_{k(j)}$ is the k^{th} parent, nested within the j^{th} site, $T_{l(k,j)}$ is seedling, nested within parent and site, an E_{ijklmn} is the residual of the m^{th} observation of the l^{th} seedling of the k^{th} parent, from the j^{th} site. The date of inoculation did not have a significant effect, and so it was not included in the model.

Heritability of pathogen susceptibility on detached leaves was calculated as:

$h^2 = 3V_{Sib} / V_T$

where V_{Sib} is the estimate of the variance component due to shared parent, and V_T is total variance. The factor 3 arises from 1/r, where r is the covariance coefficient for an equal mix of half- and full-siblings in each family group. A. Nettel and R. Dodd and found no significant inbreeding in tanoak populations (Chapter 3) so inbreeding was considered to be negligible.

To check for outliers, the image of any leaf for which Cook's $D > 4/N$ was examined individually and its measurements were repeated. Leaves were excluded from analysis in the case of a demonstrably failed inoculation (i.e., if it was noted on the original data sheet that the mycelial plug fell off the leaf during incubation), if a large portion of the lesion was due to a contaminant other than *P. ramorum*, or if leaf labels were illegible. If there was otherwise no reason to exclude the measurement the leaf was retained, with its original measured values. Out of 3697 leaves, 64 were excluded.

Analysis of Seedling Inoculation Assays

Survival and infection rate were modeled with a generalized linear model using binomial errors and a logit-linear link in the R statistical framework (R Development Core Team 2009)

with the lme4 package (Bates & Maechler 2009). The following model was chosen by iterative deletion:

$y_{lm} = \log(\pi/(1-\pi)$

$y_{lm} = \mu + P_l + \beta_l (D_{lm} - \bar{D}_l) + E_{lm}$

where the first equation represents the linear link of π, the proportion dead. y_{lm} is the linear dependent variable, P_l is the l^{th} parent, β_l is the slope for the l^{th} parent family, $D_{lm} - \bar{D}_l$ is the difference of the diameter of the m^{th} seedling from its family mean, and E_{lm} is the residual for the m^{th} seedling of the l^{th} family. The models were performed twice, once with all effects fixed in order to generate estimates, and as generalized linear mixed models with random Parent to extract variance components.

Neither mortality nor stem lesion length followed a Gaussian distribution, so heritability of response to the seedling assay is more complex. Variance components may not be directly extracted from the generalized linear models, because the validity of estimates for quantitative genetic parameters relies heavily both on the normal distribution of phenotypes and the assumption that phenotype is a result of many genes of small effect, as well as an environmental component (Simms & Rausher 1992). Although the phenotype values of binary traits will necessarily not be normally distributed, it is possible for a binary trait to be the result of an underlying genetic normal distribution if the character is a threshold trait. Threshold traits are only expressed once the underlying continuous, Gaussian, but invisible genetic liability reaches a particular level. Under a threshold model, heritability estimated from the variance components of a generalized linear model will have a non-linear relationship to the true underlying heritability, and will perform especially poorly if the trait's population-wide incidence is less than 0.2 or more than 0.8 (Van Vleck 1971, Lynch & Walsh 1992). Lynch & Walsh recommend a

conversion of heritability on the observed, binary scale to the underlying scale, by first finding h^2_o from a variance-component estimate of the additive genetic variance divided by the observed variance associated with the population-wide incidence, rather than a model estimate, and then transforming h^2_o to the underlying h^2, based on the properties of a normal distribution. Neither mortality nor lesion severity adequately fit the assumptions for threshold characters, so h^2 in the quantitative genetic sense could not be calculated.

Field Trial

In January 2008, 800 year-old seedlings from 50 families from the 2006 collection were transplanted at 1 m intervals into 10 different blocks in a *P. ramorum*-infested mixed evergreen forest in the Santa Lucia Preserve, Carmel Valley, CA. Four seedlings per family were randomly placed in each of 4 of the blocks, for a total of 16 seedlings per family. Each block was situated under cover of a symptomatic *Umbellularia californica* tree, to ensure a source for inoculum. Seedlings were assessed twice yearly between January 2008 and November 2009 for symptoms and mortality. Once each year, samples of symptomatic or dead seedlings were tested for the presence of *P. ramorum* using with a TaqMan polymerase chain reaction protocol (Hayden et al. 2006, Chapter 2, this volume; with the addition of bovine serum albumin to the PCR reaction) or isolation by culture (Erwin & Ribeiro 1996). One sample of uninfected lettuce was concurrently processed for extraction and PCR for approximately every 6 seedling samples, as a negative control; a positive control of 6 $x10^{-4}$ ng/µl *P. ramorum* DNA was included with each PCR run. To minimize the effects of sampling on disease progression, samples were not taken if doing so would remove the entire lesion, or if *P. ramorum* had previously been isolated from the seedling.

In late 2008 and early 2009 approximately 50% of the seedlings in 4 out of 10 plots suffered severe damage from small mammals, consistent with rabbit herbivory. All seedlings were caged with wire mesh to prevent further damage, and re-growth of herbivorized seedlings was monitored. Of the 161 seedlings most severely damaged, only 20 did not re-grow by November 2009, and were counted as dead. These 20 seedlings were excluded from further analyses, because their deaths can reasonably be assumed to be due to herbivory (although we cannot completely rule out other causes, including disease).

Field mortality was analyzed by a logistic fit of the proportion of seedlings dead as of the November 2009 sampling time using the package lme4 in R. Effects were chosen by iterative deletion, with the final model:

$y_{ijkl} = \log(\pi/(1-\pi)$

$y_{ijkl} = \mu + B_i + R_j + P_k + BR_{ij} + E_{ijkl}$

where μ is the grand mean, B_i is the i^{th} block, R_j is the binomial condition for a prior isolation of *P. ramorum*, P_k is the k^{th} parent family, BR_{ij} is an interaction term for block x *P. ramorum* isolation status, and E_{ijkl} is the residual of the lth observation of the kth family, $j = 1$ or 0, with or without a prior *P. ramorum* isolation, in the ith block.

We were interested in the effects of individual families, so family was modeled only as a fixed effect. Because of space constraints in the original design, families were not fully replicated across blocks, so interactions could not be not tested at this time. With increasing mortality, individual contrasts may be used to find family by block interactions.

RESULTS

Establishment of a Common Garden

Of the 12649 seeds sown, 7162 germinated with varying family-level rates from 3.5% to 91% (Table 1). Germination was highest for populations geographically close to the planting site and for those populations collected later during the harvest period with accordingly shorter periods of time in storage. Nonetheless, in 2008 the population with earliest collection dates (SM) had the highest germination success of any site at any date. The lowest germination was the BL collection in 2006. We suspect the poor germination was due to a combination of a relatively longer time in storage than others in the same cohort and considerable mold growth from excess moisture during storage.

The variable germination combined with uneven acorn production in 2006 created an unbalanced design of families within sites. We opted to include as many replicates as possible from all sites, because of the exploratory nature of the first collections and assays, despite extremely low replication in MD and BL: MD had only 3 families with enough seedlings to test, while there was a single BL family. Maximum likelihood and restricted maximum likelihood modeling were used to compensate for uneven replications, and models for the 2006 cohort are reported with and without the under-represented sites.

Inoculation Assays

Relative lesion growth in detached leaves (lesion length/leaf length) was continuously distributed and right skewed, with lesions ranging from 5% - 100% of a leaf's length in the 2006 cohort and 10%-97% in 2007 cohort. Variances were not significantly different across parent

groups within years (Bartlett test: 2006, P = 0.75; 2007, P = 0.36). With BL and MD included, variance due to collection site comprised 8% of the total in 2006, compared to 32% of the variance residing within parents and seedlings together. When BL and MD were excluded, the variance component for site dropped to near zero, but the estimates for parent and seedling effects remained constant. About 5% of the total variance in leaf susceptibility each year was due to parent (Table 2). Heritability of lesion growth was estimated at 0.16 for the 2006 cohort assay and 0.15 for the 2007 cohort assay.

The mean relative lesion length for site MD was significantly greater than those of all the other populations, after correction for multiple comparisons by Tukey's HSD (Fig 1), but the difference may well be an artifact of the extremely small sample size. When MD and BL were eliminated from the analysis, the fixed effects for site remained statistically significant overall, and sites LP and SM were significantly different from each other.

There were significant differences among the most extreme parent means each year. The means with a simplified version of the Tukey's HSD are depicted in Fig. 2

There was no significant relationship between family means (P = 0.26) or ranks (P = 0.33) for the two different inoculation assays within the 2007 cohort. After 10 months, lesions in seedling stems had the same range they did in detached leaves, from 0% to 100%, but with a decidedly non-normal distribution (Fig 4). Seedling diameter and shared parent had significant (P < 0.01) effects on lesion length and survival (Table 4). Four families (BL-1, BL-4, BL-13, and BL-25) had significant negative effects lesion length (P < 0.02). To make multiple comparisons a simple linear analysis of variance was performed on the logit-transformation of lesion/stem length; by this test all five families' effects estimates were significantly different from those of the two most susceptible families, BL-19 and BL-24, by Tukey's HSD.

Two families, BL-21 and BL-25 experienced 100% survival. Two others, BL-4 and BL-13, exhibited significant increased probabilities of survival, while BL-19 and BL-24 had the greatest mortality (Fig. 5). The presence or absence of infection did not differ significantly among families (not shown); absence of infection ranged from 0% to a maximum of 22%, in family BL-1.

The heritability estimates for mortality and relative lesion length were so high as to be meaningless. If h^2 were calculated as for a linear model, each trait would have estimates greater than 1 (Table 5). Therefore, the correction for threshold characters outlined by Lynch & Walsh (1998) was not valid.

Field Trial

As of November 2009, 82% of seedlings transplanted into the infested field site had survived. Overall mortality rate was 18%; with the clear cases of herbivory excluded, mortality was 16%.

Non-fatal herbivory had a protective effect against disease. Of the surviving plants in November 2009, 76% of the zero-to-moderately herbivorized seedlings had some disease symptoms in 2009, while 7% of the 141 surviving, severely herbivorized seedlings were symptomatic (Likelihood ratio $X^2 = 259$, $P < 0.0001$). Out of 228 seedlings sampled in May 2009, there were 60 detections of *P. ramorum*, by PCR, culture, or both. Fifty-eight were positive by PCR. These were equally distributed among samples from living and dead tissue (likelihood ratio $X^2 = 1.198$, $P = 0.55$). There were 10 failed amplifications (negative results both by the *P. ramorum*-specific primers and the universal primers, which amplify all eukaryotics, including the host tissue, as an internal positive control), a failure rate of less than 5%. All

lettuce-negative controls were negative by specific primer sets, and all PCR water controls were negative by both specific and universal primer sets.

We found significant effects of family, seedling diameter, *P. ramorum* detection, block, and a block-by-*P. ramorum* detection interaction on field mortality, modeled as following a binomial distribution using the lmer function in R (Table 6). Interestingly, the family with the highest mortality in the field was BL-24, which also had the highest mortality after tip inoculation. It was the only family represented in both 2006 and 2007 cohorts, so it was the only family for which there are tip inoculation results included in the field trial, so the observation has only anecdotal value. The two families with the highest resistance by the detached-leaf assay (SM-29 and SM-74) had significantly and marginally significantly less field mortality, respectively, in contrast to BL-24.

DISCUSSION

The collections and initial resistance assays we describe here establish a population of tanoak seedling families for genetic analysis and a methodology for phenotypic assessment of susceptibility to *Phytophthora ramorum*. Two additional, more-targeted cohorts are currently in production, and will be tested in turn. Future collections, assays, and collections can be designed iteratively, based on information from the populations currently in hand, and challenged against top performers from prior years.

We found considerable family-level variation in susceptibility by both detached leaf and intact seedling inoculations, although with different messages from each assay. Detached-leaf susceptibility had a continuous distribution and $h^2 = 0.15$ in each trial, in line with measured heritabilities for quantitative disease resistance and other morphological traits targeted for

selection in forest tree breeding, which generally range from 0.1 – 0.3 (Cornelius 1994, Carson & Carson 1989, Scotti et al. 2010). Susceptibility varied significantly with source population, regardless of whether the two source sites with low family replication were included in the fixed model, but almost all of the mixed-model site variance was contributed by the 3-family MD population. MD was the only site included from the northern coastal California region in these first studies; a repeated collection would be warranted to discern if the population differentiation is real or a sampling artifact.

Excluding MD, there was less population-level structure to susceptibility in this common garden study than we found in detached leaves from natural populations or than Dodd and colleagues (2005) found in stem cuttings of *Q. agrifolia* tested with the same pathogen. The reduction in structure points to environmental factors as the primary driver behind the small amount of population structure we did see.

The differences among populations were statistically significant but not large, and may have been as simple as a sampling problem, or differences in maternal environment. Despite the common garden design, environmental variation across sites is likely to have played a role in seedling disease susceptibility. Controlled-cross designs using wild trees as parents would be so resource-intensive as to be impractical, so until there is a cultivated population of reproductive age, sibling studies of tanoak will necessarily use open-pollinated seed. In keeping with tree-breeding convention, we used the notation for narrow-sense heritability for our estimate, but sibling designs with a maternal shared parent introduce maternal effects to the shared-family variance component; moreover, the likely presence of full siblings introduces dominance interactions (Falconer & Mackay 1996).

Maternal effects, in particular, are a potential complication. Maternal effects can influence any number of characters, up to and including induced defenses (Hoelski 2007). Most straightforwardly, the amount of energy allocated to seed mass will directly influence offspring vigor. Oaks and tanoaks are masting species, producing temporally heterogeneous seed crops (McDonald & Tappeiner 2002). Koenig and his colleagues have documented both (i) that California red oaks' masting patterns depend partly on local environmental variation, with greater acorn production after years with good growing conditions (Koenig et al. 1996,), and (ii) that greater seed size is correlated with increased seed production (Koenig et al. 2009). The size of tanoak seed reserves significantly affect the biomass and photosynthetic rates of the resulting seedlings (Kennedy et al. 2004), and acorn mass in Chinese oaks was shown to be positively correlated with germination rates and tolerance of seed herbivory (Xiao et al. 2007). The near-perfect accord of detached-leaf susceptibility rank with germination rank (Table 1, Fig 1) is not likely to be a coincidence; maternal resources almost certainly contributed some portion of the observed susceptibility. However, synchronous masting patterns are near constant at spatial scales of up to 10 km (Liebhold et al. 2004), so to the extent that they are driven by local climate, a good deal of these maternal resource effects should be manifested as environmental, rather than genetic, effects.

Seedling mortality ranged from 0% to 64%, and 49% of the stem lesions extended the full length of the stem. There were strong significant effects of family and stem diameter on lesion development and survivorship, however, neither could be modeled as a quantitative genetic threshold trait. The primary causes of bimodal or trimodal distributions are either a gene of major effect, or a large environmental effect (Falconer & Mackay 1996). The observed pattern is intriguing and merits further study.

Disease incidences did not differ significantly among families, however, major gene effects need not confer only complete resistance. Given the degree to which tanoaks are susceptible to *P. ramorum* in the field, it would be extremely surprising to discover major genes for complete resistance at any frequency, and especially on the first sampling. Mechanisms to slow or limit disease also may be inherited via major genes. Some forms of partial resistance in the white pine blister rust system (Kinloch et al. 2008) may be monogenically inherited and there is molecular evidence that host R genes and pathogen effectors can interact to result in only partial suppression of the pathogen (Bent & Mackey 2007).

Developing clonal propagation techniques for tanoak should be a high priority. Tanoak seed production is heterogeneous and, unlike conifer seeds which may be collected in the thousands and kept in long-term storage almost indefinitely, acorns cannot be stored (McDonald & Tappeiner 2002). The initial phases of the project focused on a single pathogen strain, and only one of each common garden and field sites. The availability of clonal replicates would greatly facilitate the study of genotype x environment and host x pathogen interactions, and would facilitate resistance assays that do not kill the subject. Seedling cuttings cannot be used as a substitute for intact seedlings for inoculations; their lesions tend to be larger, are normally distributed, and have a much higher disease incidence (K Hayden, unpublished data). That fact in itself is interesting, and should be further studied.

Susceptibility ranks based on the two inoculation assays were uncorrelated. The lack of correlation does not necessarily imply that one assay is incorrect or not meaningful. Host resistance may incorporate several different traits, which may or may not be correlated with each other (Carson & Carson 1989). Western white pines exhibit both a hypersensitive response as well as other, quantitatively measured, types of resistance to white pine blister rust, such as

delayed or reduced stem symptom development, and both types can contribute to survival in natural field settings (Kinloch et al. 2008, Kegley & Sniezko 2004, Sniezko 2006). Likewise, some Port-Orford-cedar (*Chamaecyparis lawsoniana*) trees resistant to *Phytophthora lateralis* stem inoculations are susceptible to foliar inoculations (Oh et al. 2006).

To study natural disease dynamics we have established a common garden of seedlings transplanted to an infested forest. This garden will allow long-term validation of laboratory inoculation assays, which is critical for understanding whether the differences in lesion development observed in the lab are biologically relevant. With only two years elapsed and 18% mortality, the trial is in its early stage [field studies often go seven years or more (Kinloch et al. 2008, Sniezko 2006, Yang et al. 1998)], yet we found evidence of *P. ramorum* infection of the study trees: 60 isolations of the pathogen, and, despite the low mortality, a significant effect of *P. ramorum* isolation status on death.

Each of the pieces we describe here– the establishment of a Berkeley, CA study population, the development of two complementary assays for disease susceptibility, and the initiation of a long-term field trial—set the stage for studies of host-pathogen interactions and genetics in a system of immediate, practical need. We found signs that tanoaks do possess heritable genetic variation in resistance to *P. ramorum*; the test populations and garden sites we established provide a mechanism with which to begin to test the potential for this resistance to prevent extirpation of a unique species.

ACKNOWLEDGEMENTS

Sincere thanks go to Richard Sniezko and Susan Frankel for conceiving of and fostering the tanoak resistance and genetics program, and to Perry Joiner, Alex Lundquist, Jessica Wright,

Annie Mix, Barb Rotz, John Andrews, Richard Dodd, Alejandro Nettel, Ellen Simms, Cindy Roessler and her army of volunteers, and each and every member of the Garbelotto Lab. The USDA-Forest Service PSW, the Mid-Peninsula Regional Open Space District, and the Point Reyes National Seashore generously provided funding.

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