TaqMan Chemistry for *Phytophthora ramorum* Detection and Quantification, with a Comparison of Diagnostic Methods

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ABSTRACT

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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 17 other *Phytophthora* spp. tested was amplified by this assay. A comparison of the nested and non-nested TaqMan assays, and of one other nested assay, showed nested methods to be significantly more sensitive than nonnested and showed 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, whereas the single-round TaqMan protocol detected significantly fewer positive samples. Finally, we documented increases in the quantity of pathogen DNA in *Umbellularia californica* leaves in initial stages of infection.

Additional keywords: quantitative PCR, real-time PCR, sudden oak death.

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* spp. 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. (37) 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 often have 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 polymerase chain reaction (PCR) methods can be used to detect the organism of interest as well as to quantify the organism's DNA (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 to 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 then may 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. Although 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 methods' 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). Methods of detecting *P. ramorum* have proliferated; however, only limited comparative data are available to evaluate sensitivity of TaqMan versus non-TaqMan and of nested versus single-round approaches. Although sensitivities can be calculated theoretically and tested using DNA isolated from pure culture, the substrate from which detection is attempted can introduce enormous variability in assay sensitivity (23,35,36). 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 to (i) develop a reliable, quantitative method for detecting *P. ramorum* in plant tissue; (ii) compare the sensitivity of this method with two others currently available; (iii) use the assay to detect the pathogen in samples collected from California woodlands; and (iv) determine whether this method can be used to quantify the pathogen DNA in infected plant tissue.

MATERIALS AND METHODS

Assay specifications. *P. ramorum*-specific primers (Pram5 and Pram6) and an internal dual-labeled fluorogenic (TaqMan) probe (Pram7) (Table 1; Fig. 1) were designed within the internal tran-

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scribed spacer region 2 (ITS2) of the ribosomal (r)DNA 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 (Table 1; Lt1UnivPrimer, RtUnivPrimer, and UnivProbe) 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 used further 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* spp., 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); and those which frequently are co-isolated with *P. ramorum*, such as *P. nemorosa* and *P. pseudosyringae* (11,25,38).

Deoxyribonucleic acids were extracted from lyophilized tissue using a cetyltrimethylammonium bromide (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 of 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 of ultrapure water (nanopurified, autoclaved, and UV irradiated) and then were stored at –20°C in 0.1× Tris-EDTA buffer. Unless noted otherwise, extracts from pure mycelia were diluted 1:1000 in ultrapure water prior to amplification, whereas 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 (0.05 M KCl, 0.01 M Tris at pH 8.3, gelatin at 0.1 mg/ml, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.5 µM each primer Phyto1 and Phyto4, 0.01 µM each Lt1UnivPrimer and RtUnivPrimer, 1.25 U of Taq Polymerase) (Promega Corp., 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; and a final extension at 72°C for 10 min. Ramp rate was 3.3°C/s heating and 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× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 0.2 µM each primer, 0.2 µM each probe, and 5 µl of template DNA. PCR was performed in an

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[a]
Phyto1[b]	CATGGCGAGCGCTTGA	687	ITS1, 5.8S, ITS2
Phyto4[b]	GAAGCCGCCAACACAAG	…	...
Pram5	TTAGCTTCGGCTGAACAATG	73	ITS2
Pram6	TGACTGGTGAACCGTAGCTG	…	...
Pram7	(6-FAM)ATGCTTTTTCTGCTGTGGCGGTAA(BHQ1a-6FAM)	…	...
Lt1UnivPrimer	TTGGAGGGCAAGTCTGGT	82	SSU
RtUnivPrimer	TTGTTGCAGTTAAAAAGCTCG	…	...
UnivProbe	(5HEX)CCGCGGTAATTCCAGCTCCAATAG(BHQ1a-5HEX)	…	...

[a] ITS = internal transcribed spacer; SSU = small subunit.
[b] Hayden et al. (12).

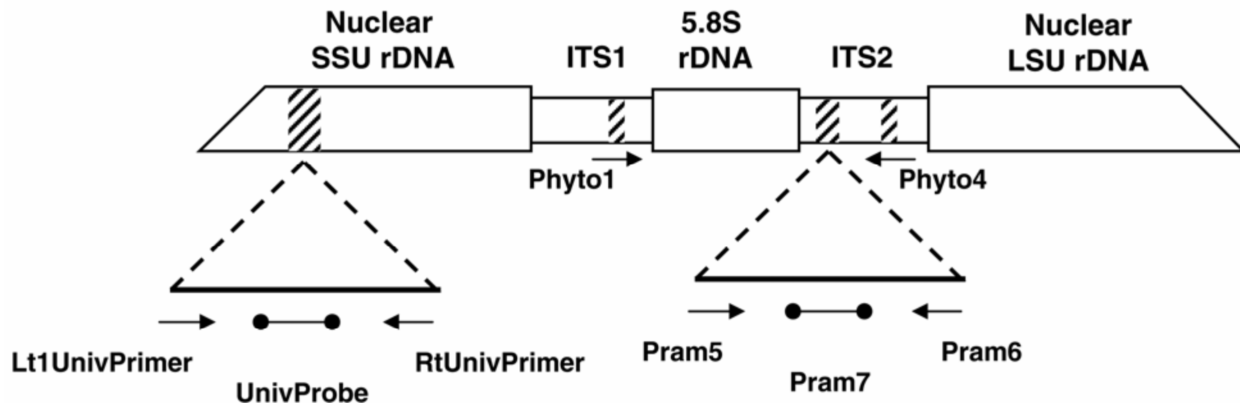

Fig. 1. Relative locations of primers and fluorogenic probes within the nuclear ribosomal DNA region (not to scale). Hatched regions represent sites of specific binding. Primers are indicated with single-headed arrows and probes with double-headed bars. ITS, internal transcribed spacer; LSU, large subunit; SSU, small subunit.

iCycler IQ thermalcycler using the following conditions: 1 cycle at 50°C for 10 min, 1 cycle at 95°C for 3 min, 40 cycles at 95°C for 15 s, and 60.5°C for 1 min. Ramp rate was 3.3°C/s heating and 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 also were compared with a nested protocol using the intercalating dye SYBR green for detection (12). Asymptomatic leaves of *Rhododendron macrophyllum* and *L. 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 because 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 them tip-first into a 1×10^4 zoospore/ml suspension for 1 min. Leaves were incubated at ambient temperature (10 to 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 of sterile water for 48 h. Water then was poured off and the leaves were incubated further for 5 days. Fourteen days after inoculation, the leaves were washed with deionized water and 6-mm discs, each containing approximately equal portions of 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 10 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 1 h at room temperature, zoospores were counted with an hemacytometer and then diluted appropriately to working concentrations.

For each species inoculated, 30 samples—each consisting of two discs—were placed in 2-ml centrifuge tubes and DNA was extracted as described previously. Infection (or lack thereof in uninoculated leaves) was confirmed by plating 15 discs from inoculated and uninoculated leaves of each plant species onto P_{10}ARP *Phytophthora*-selective agar (per liter: 17 g of corn meal agar, 0.25 g of ampicillin, 0.4 ml of 2.5% Pimaricin, 0.01 g of rifampicin in 1 ml of dimethyl sulfoxide, and 5 ml of 0.5% pentachloronitrobenzene in ethanol).

TABLE 2. Isolates of *Phytophthora* spp. used to determine specificity of reaction of the *Phytophthora ramorum*-specific primers and fluorogenic probe, using a nested protocol[a]

Species	Isolate no.[b]	Host	Origin
P. boehmeriae	325[PT]	*Boehmeria nivia*	Papua New Guinea
P. cambivora	PDR198513[CB]	*Quercus agrifolia*	California, United States
P. capsici	302[PT]	*Capsicum annuum*	Florida, United States
P. capsici	P141[DR], 3300[GB]	*Lycopersicon esculentum*	
P. cinnamomi	P6379 (A1)[MC]	*Ananas comosus*	Taiwan
P. cinnamomi	P6379(A2)[MC]	*Persea americana*	California, United States
P. cryptogea	IMI 045168	*L. esculentum*	New Zealand
P. erythroseptica	355[PT]	*Solanum tuberosum*	Maine, United States
Phytophthora sp.	9C[CB]	*Ardisia japonica* 'Chirimen'	California, United States
Phytophthora sp.	18[CB]	*Photinia* × *fraseri*	California, United States
P. gonapodyides	393[PT], NY353[WW]	*Malus sylvestris*	New York, United States
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, United States
P. lateralis	PL16[DR]	Soil	California, United States
P. lateralis	PL27[DR]	*Taxus brevifolia*	California, United States
P. lateralis	PL33[DR]	*Chamaecyparis lawsoniana*	California, United States
P. megasperma	309[PT], 336[PH]	*Pseudotsuga menziesii*	Washington, United States
P. nemorosa	P16[DR]	*Umbellularia californica*	California, United States
P. nemorosa	P44[DR]	*U. californica*	California, United States
P. nicotianae	P1352[MC], 331[PT]	*Nicotiana tabacum*	North Carolina, United States
P. palmivora	P1-10[DJM]	*Theobroma cacao*	Costa Rica
P. pseudosyringae	P40[DR]	*Q. agrifolia*	California, United States
P. syringae	PDR115773A[CB]	*Rhododendron* sp.	California, United States
P. ramorum	Pr-01[DR], CBS110534	*Q. agrifolia*	California, United States
P. ramorum	Pr-06[DR], ATCC MYA-2435	*Q. agrifolia*	California, United States
P. ramorum	Pr-13[DR]	*Q. agrifolia*	California, United States
P. ramorum	Pr-36[DR], CBS110953	*Q. agrifolia*	California, United States
P. ramorum	Pr-52[DR], CBS110537, ATCC MYA-2436	*Rhododendron* sp.	California, United States
P. ramorum	Pr-72[DR], CBS110954	*Rhododendron* sp.	California, United States
P. ramorum	Pr-102[DR], ATCC MYA-2949	*Q. agrifolia*	California, United States
P. ramorum	Pr-105[DR]	*Lithocarpus densiflora*	California, United States
P. ramorum	Pr-106[DR], CBS110956	*U. californica*	California, United States
P. ramorum	Pr-108[DR]	*U. californica*	California, United States
P. ramorum	Pr-114[DR]	*U. californica*	California, United States
P. ramorum	Pr-120[DR]	*L. densiflora*	California, United States
P. ramorum	Pr-159[DR], CBS110543	*L. densiflora*	California, United States
P. ramorum	Pr-SDC21.6[DR]	*Sequoia sempervirens*	California, United States
P. ramorum	BBA 12/98[SW], CBS101551	*R. catawbiense* 'Grandiflorum'	Germany
P. ramorum	Phyram1[EM]	*R. catawbiense* 'Grandiflorum'	Mallorca, Spain

[a] *P. ramorum* was the only species amplified by the single-round or nested TaqMan assay.

[b] Isolate numbers on the same line are synonymous. Isolate source: CB, Cheryl Blomquist; GB, Greg Browne; MC, Mike Coffey; PH, Phil Hamm; EH, Everett Hansen; DJM, Dave Mitchell; EM, Eduardo Moralejo; PT, Paul Tooley; DR, David Rizzo; SW, Sabine Werres; WW, Wayne Wilcox.

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 noninoculated 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 noninoculated controls, and 20 ultrapure water controls were distributed randomly across a 96-well PCR plate for each run. There were four 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 to 20 days before second-round PCR was performed.

Positives for single-round and nested TaqMan PCR were determined using threshold cross times (threshold cycle [Ct]). If the sample's Ct was ≤40 cycles it was scored as positive. This threshold was selected because, at times, nonspecific 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 (version 5.01; 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). In all, 35 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. In our experience, this method does not adequately extract or purify DNA from wood tissue; therefore, 310 wood or bark samples were processed using a QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA).

qPCR. 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 nine trees; three individuals of low, three of intermediate, and three of high resistance to *P. ramorum*, as determined by size of lesion 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 three trees, one each of high, medium, or low resistance. Leaves were inoculated by inserting the leaves tip first into 50-ml conical polypropylene tubes. A zoospore suspension (300 µl, 2×10^4 zoospores/ml) of *P. ramorum* isolate Pr52 (CBS110537, ATCC MYA-2436) was pipetted 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 h, 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 1,000-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. Although 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 0.1× Tris-EDTA buffer to a 10-fold series ranging from 0.0001 to 1 ng μl^{-1}. The lowest quantity of *P. ramorum* that can be amplified reliably by a single round of amplification with this method is 0.00001 ng μl^{-1} (or 50 fg/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 then was 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 subsequently was 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 allowed 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. From each of 60 extractions randomly chosen from the experimental lot, 5 µl were pooled together, and the DNA concentration of this bulked sample

was determined by UV spectrometry. The bulk sample was diluted in 0.1× Tris-EDTA buffer to create a 10-fold dilution series, ranging from 0.0001 to 10 ng μl^{-1}. Three replicates of each concentration were amplified with the Univ 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 *P. ramorum* DNA by the measured concentration of all 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 (version 5.01; SAS Institute) to compare ratios across both trials by ANOVA, where effects tested were incubation time, trial, and their interaction. For trial 1 only, we also tested for the effect of individual tree nested within resistance category, with tree as a random effect, and the interaction; and for trial 2 alone, we also tested the effect of individual tree as a random effect, along with the tree by incubation time interaction. Because some data points had been discarded, the design was unbalanced; therefore, analyses with random effects were performed using the restricted maximum likelihood method. For any significant effect, we performed a Tukey test in JMP to compare means among groups.

RESULTS

P. ramorum was detected by the Pram set using the nested protocol in quantities as low as 15 fg. No other *Phytophthora* sp. 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).

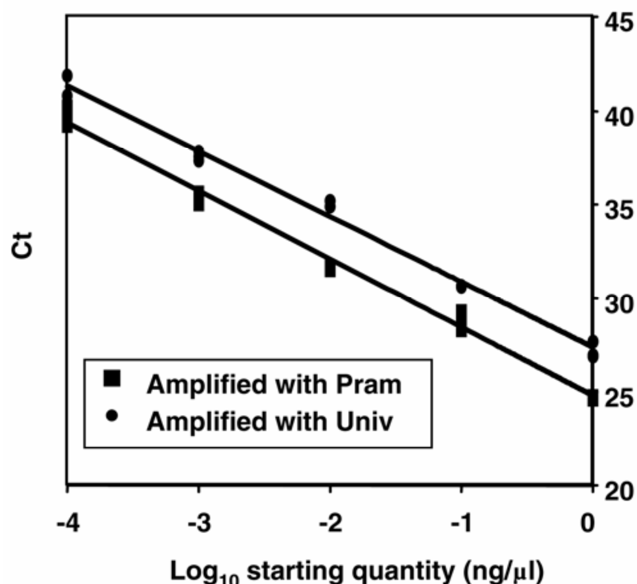

Fig. 2. Standard curves for quantifying *Phytophthora ramorum* within infected tissue. With every polymerase chain reaction run, curves were calculated for both *P. ramorum*, using known quantities of *P. ramorum* DNA spiked with extract from uninfected host leaves and amplified with the *P. ramorum*-specific 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 = 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.

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 detected more easily in leaves of *R. macrophyllum* than in *L. densiflora*. In *R. macrophyllum*, both nested methods detected the pathogen in eight of eight least-diluted samples in all four replicate runs. At this dilution and in this host, the single round of

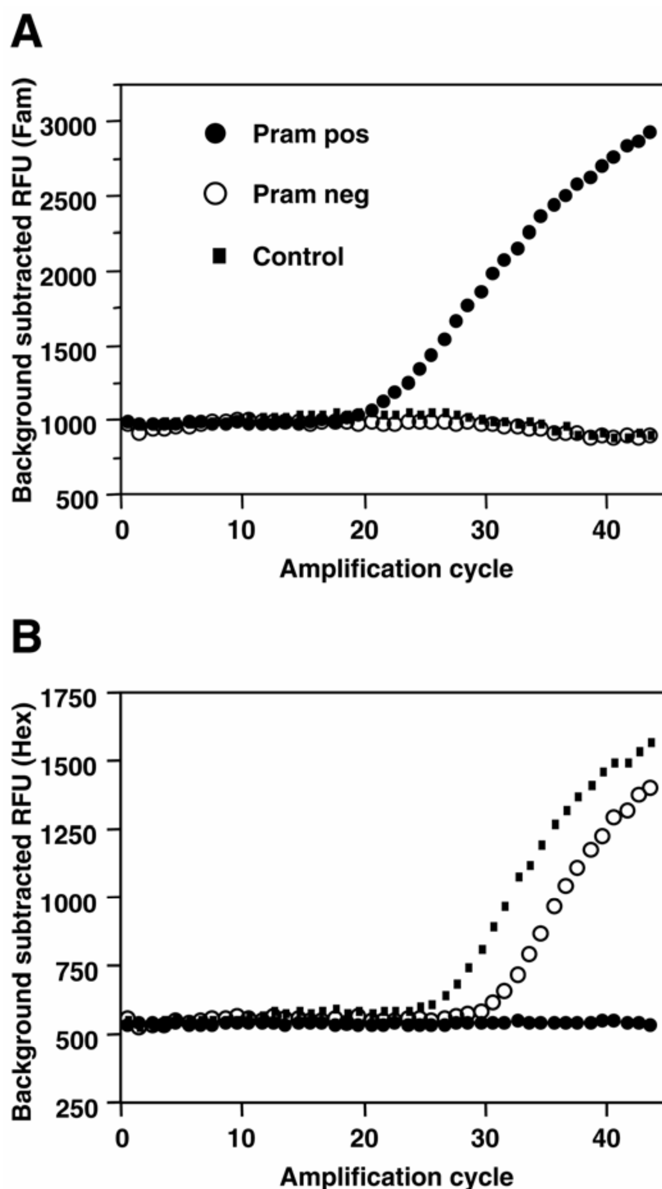

Fig. 3. Interference of UnivProbe reporting by the Pram7 product. Each multiplexed TaqMan polymerase chain reaction generates two amplification curves, one from each fluorophore's excitation and emission filters; RFU = relative fluorescence units. **A,** Leaf tissue infected with *Phytophthora ramorum* shows fluorescence above a baseline threshold using the Fam-labeled probe Pram7 (closed circles, Pram pos), whereas 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. **B,** 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.

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 honestly significant difference [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).

TABLE 3. Effect tests by analysis of variance on detection frequency of *Phytophthora ramorum* in four replicate polymerase chain reaction (PCR) runs[a]

Source	df	SS	MS	*F* ratio	*P* > *F*
Host	1	2.217	2.217	60.819	<0.0001
Dilution	2	5.077	2.539	69.650	<0.0001
Method	2	0.847	0.423	11.617	<0.0001
Method × dilution	4	0.366	0.092	2.513	0.0481
Error	80	2.916	0.036	…	…

[a] Two plant hosts were tested, as well as three detection methods (nested and nonnested TaqMan PCR, and nested PCR with SYBR Green detection) at three dilutions of DNA extracted from infected hosts. All dilutions were supplemented to contain standard amounts of plant DNA extracts.

Environmental application. 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.

qPCR. 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

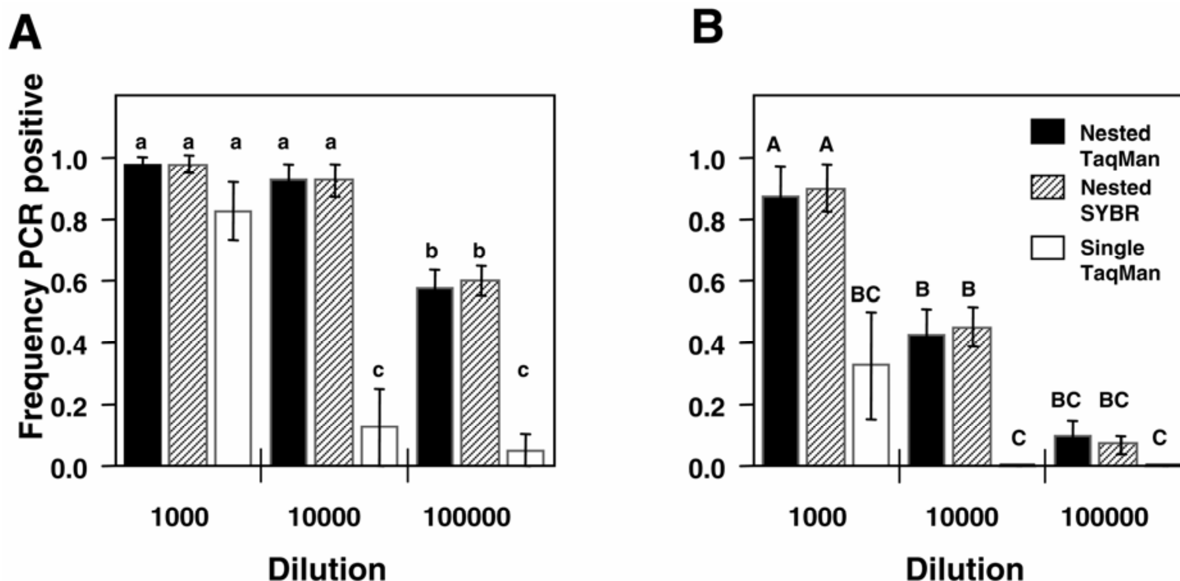

Fig. 4. Relative success in detecting *Phytophthora ramorum* from infected leaves by the nested TaqMan method presented here, a single round of TaqMan amplification without preamplification, and a nested polymerase chain reaction (PCR) method using SYBR green for detection. Bars connected by the same letter were not significantly different at $P = 0.05$; data were analyzed within each host, but not across. **A,** Leaves of *Rhododendron macrophyllum* and **B,** leaves of *Lithocarpus densiflora*. 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.

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 total variance
Trials 1 and 2						
Days	2	136.866	26.543	<0.0001	...	...
Trial	1	0.10433	0.0405	0.8407	...	...
Days × 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 × susceptibility	4	7.263	0.412	0.7965	...	...
Tree [susceptibility] (random)	6	4.885	...	...	0.0581	1.290
Days × tree [susceptibility] (random)	11	3.357	...	...	0.0417	0.925
Error	111	489.032	...	...	4.4060	97.785
Trial 2						
Days	2	45.147	27.048	0.0047	...	...
Tree (random)	2	74.966	...	...	0.516	36.689
Days × 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).

from a mean of 1.1% ($n = 30$) at 24 h to a mean of 5.1% ($n = 54$) at 48 h and 4.3% ($n = 53$) at 72 h. The mean at 24 h was significantly different (Tukey's HSD, $P < 0.03$) from those at 48 and 72 h, which were indistinguishable (Fig. 5). There was no significant effect of trial or treatment–trial interaction. However, in trial 2, there was a significant difference between the mean at 48 h (2.9%, $n = 62$), and the mean at 72 h (4.8%, $n = 68$). Resistance category was not significant at the level of $P < 0.05$ in trial 1, and the effect of individual *U. californica* trees contributed very little to the overall variance. However, in trial 2, the effect of individual tree accounted for nearly 37% of the total variance. The tree that previously had 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 h but not at 24 or 48 h (Tukey's HSD, $P < 0.05$).

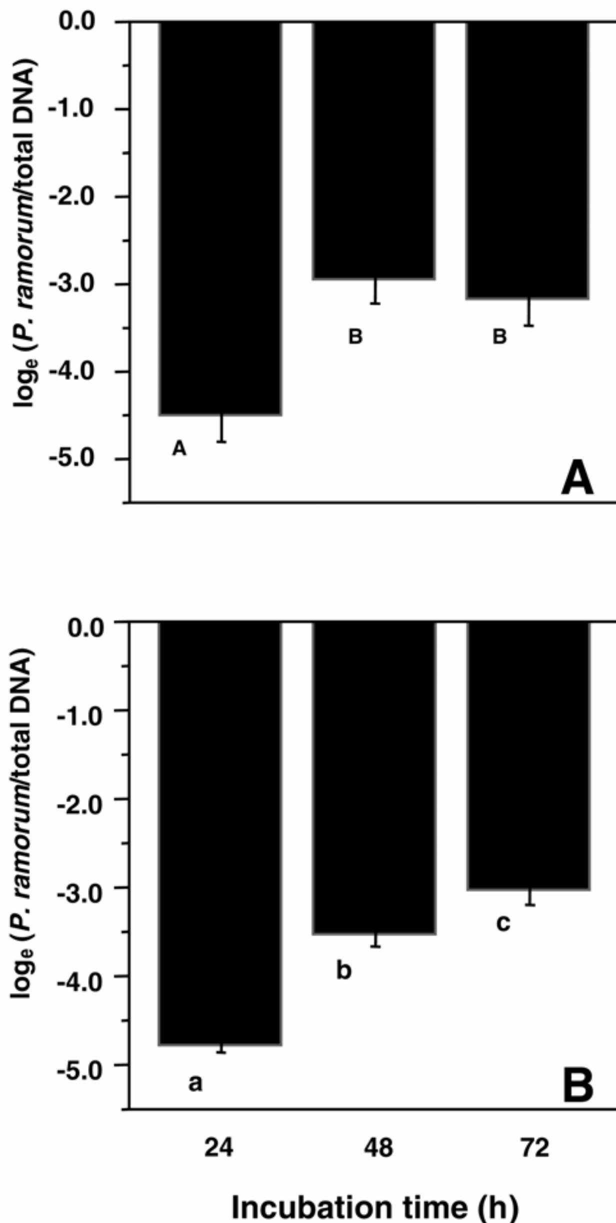

Fig. 5. Quantity of *Phytophthora ramorum* in infected *Umbellularia californica* leaves during the early stages of an infection, represented as a proportion of the total DNA in each sample. Groups significantly different from each other at $P = 0.05$ within each trial are marked with different letters;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

DISCUSSION

The real-time PCR method presented here not only is a sensitive and specific method for the diagnosis of *P. ramorum* in planta but also can 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* spp. 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), whereas 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* (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 1 to 3 days of inoculation. Lesion expansion rate is one measure by which host resistance to pathogen infection often is assayed (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 h after exposure to zoospores, despite the absence of

significant visible disease symptoms, may be used to design rapid tests aimed at differentiating susceptible and nonsusceptible plant species (e.g., interspecific comparisons). Even with relatively 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 (27), it promises to considerably expand understanding of this economically and ecologically important pathogen and its effects on a broad range of hosts.

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