Molecular Detection of *Phytophthora ramorum* by Real-Time Polymerase Chain Reaction Using TaqMan, SYBR Green, and Molecular Beacons

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ABSTRACT

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Sudden oak death, caused by *Phytophthora ramorum*, is a severe disease that affects many species of trees and shrubs. This pathogen is spreading rapidly and quarantine measures are currently in place to prevent dissemination to areas that were previously free of the pathogen. Molecular assays that rapidly detect and identify *P. ramorum* frequently fail to reliably distinguish between *P. ramorum* and closely related species. To overcome this problem and to provide additional assays to increase confidence, internal transcribed spacer (ITS), β-tubulin, and elicitin gene regions were sequenced and searched for polymorphisms in a collection of *Phytophthora* spp. Three different reporter technologies were compared: molecular beacons, TaqMan, and SYBR Green. The assays differentiated *P. ramorum* from the 65 species of *Phytophthora* tested. The assays developed were also used with DNA extracts from 48 infected and uninfected plant samples. All environmental samples from which *P. ramorum* was isolated by PARP-V8 were detected using all three real-time PCR assays. However, 24% of the samples yielded positive real-time PCR assays but no *P. ramorum* cultures, but sequence analysis of the *cox*I and II spacer region confirmed the presence of the pathogen in most samples. The assays based on detection of the ITS and elicitin regions using TaqMan tended to have lower cycle threshold values than those using β-tubulin and seemed to be more sensitive.

Since 1995, extensive oak mortality, referred to as sudden oak death (SOD), has been reported in California. In 2001, a new pathogen called *Phytophthora ramorum* Werres, de Cock & Man in't Veld, was described (60) and identified as the causal agent of SOD (12,14,20,37,45,51). This pathogen has since spread to several counties in California (14 in 2004) and was discovered in Oregon in 2001 (22,33), but is now increasing its range. It was found again in 2003 in nurseries in Oregon, Washington, and British Columbia (25), and was subsequently eradicated from infested sites. In 2004, the pathogen was discovered in nurseries in several U.S. states and one Canadian province after a nursery in California shipped infected material across the continent. *P. ramorum* is also present in Europe where it has been reported in 14 countries, mostly in nurseries, but also in public parks, where it causes leaf blight and shoot dieback mainly in *Rhododendron* sp. and *Viburnum* sp. (16,32,36,44,46,58). Recently, it was found on southern red oak, *Quercus falcata*, in England where the bleeding canker symptoms typical of SOD were observed. *Q. rubra* is susceptible to *P. ramorum* in artificial inoculations and two trees have been found naturally infected in the Netherlands (9,10,17).

P. ramorum infects a broad range of hosts and causes different symptoms, including cankers, leaf spots, blights, and diebacks (19). Inspection and proper diagnosis of nursery material can therefore be challenging especially on hosts for which the etiology has not been described. Experience in Europe and North America indicates that *P. ramorum* spreads readily on infected nursery material by zoospore release and that it may also be spread via chlamydospores in soil debris and rain splash (13).

In order to prevent the movement of material from infested to disease-free locations, state and federal quarantine measures were put into place in the United States and in Canada. Despite these measures, the disease spread to new locations along the coast of the Pacific Northwest in 2003 and 2004. Intensive surveys have been conducted in several countries, including Canada, where this pathogen could represent a risk, to determine whether it is present and to assess the extent of its distribution. Depending on jurisdiction and circumstances such as time of year, survey samples are tested by direct isolation on selective medium, or by some combination of isolation, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR). One of the difficulties encountered with identifications based solely on morphology is that it requires mycological expertise and it does not always allow the distinction of variants within species. This is important in the case of *P. ramorum* since close relatives of *P. ramorum* exist in

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North America and isolates of *P. ramorum* from North America and Europe are genetically distinct (8,29,30,35). Furthermore, seasonal variation in the ability to recover *P. ramorum* from infected field-collected tissue has been reported (27). Molecular diagnostic tools can allow rapid and sensitive pathogen detection and provide an increased confidence level in the identification, even when the pathogen cannot be isolated. Molecular assays have the advantage that they can be used on most types of material, from pure to mixed cultures of the pathogen or symptomatic to asymptomatic host tissue samples. However, identification of *P. ramorum* based solely on molecular methods can yield false positive results (18,47,48,53) or questionable results that cannot be confirmed by isolation or other methods. Thus, identification of *P. ramorum* for regulatory purposes is most reliable when using a combination of molecular methods and other methods.

Recent phylogenetic analyses using internal transcribed spacer (ITS) (11) sequences of the rRNA cistron revealed a close relationship between *P. ramorum* and *P. lateralis* Tucker & Milbrath, with a total of 12 single nucleotide polymorphisms between them (18,51). In addition, other phylogenetic analyses of *Phytophthora* species conducted with mitochondrial and nuclear DNA sequences have revealed a close relationship with *P. hibernalis* (34,38). Given the close phylogenetic relationships among these species, one PCR assay, developed from the ITS region, may not always reliably discriminate *P. ramorum* from *P. lateralis* or *P. hibernalis* (18,47). This could be important since *P. lateralis*, the causal agent of Port Orford cedar root rot, is commonly found on the west coast of North America (18,19,62). Other real-time PCR protocols were developed to detect *P. ramorum* and were using single gene detection (26,27,53,54).

The objectives and approach of this investigation were the following: (i) to sequence two nuclear genes (β-tubulin and elicitin "ramorumin") of *P. ramorum* and related species and use existing ITS sequences to identify polymorphisms specific to *P. ramorum*; (ii) to design and test reverse transcription (RT)-PCR assays to specifically detect *P. ramorum*, based on the polymorphisms identified using three reporter technologies (SYBR Green, TaqMan probes, and molecular beacons targeting the three gene regions); and (iii) to validate the real-time PCR assays using DNA from pure cultures of a comprehensive collection of *Phytophthora* species and from infected plant samples.

MATERIALS AND METHODS

Isolates. All isolates of *Phytophthora* used in this study are listed in Table 1. The *P. ramorum* collection comprised 38 isolates

TABLE 1. Isolates of *Phytophthora* species from different culture collections used in this study

Phytophthora species	Collection number[a]	*Phytophthora* species	Collection number	*Phytophthora* species	Collection number
arecae[b]	CBS 305.62	*katsurae*[b]	CBS 587.85		
boehmeriae[b]	CBS 291.29	*lateralis*[bc]	ATCC201856	*ramorum*	CBS 110544
botryosa[b]	CBS 581.69	*lateralis*	CBS 168.42	*ramorum*	CBS 109278
brassicae[b]	CBS 178.87	*meadii*[b]	CBS 219.88	*ramorum*[c]	CBS 101552
brassicae[b]	CBS 686.95	*megakarya*[b]	CBS 238.83	*ramorum*	CBS 101549
cactorum[b]	CBS 108.09	*megasperma*[b] v. *megasperma*	CBS 402.72	*ramorum*	CBS 101331
cactorum[b]	CBS 108.09	*melonis*[b]	CBS 582.69	*ramorum*	CBS 110535
cactorum[c]	DAOM BR 675	*mexicana*[b]	CBS 554.88	*ramorum*	CBS 110547
cambivora[b]	CBS 248.60	*mirabilis*[b]	CBS 678.85	*ramorum*	CBS 1110536
capsici[b]	CBS 128.23	*multivesiculata*[b]	CBS 545.96	*ramorum*	CBS 110542
cinnamomi[b]	CBS 144.22	*nicotianae*[b]	CBS 305.29	*ramorum*	CBS 110534
cinnamomi[bc]	DAOM BR 680	*nicotianae*[b] (type of *terrestris*)	CBS 109.17	*ramorum*	CBS 110900
cinnamomi[b] v. *parvispora*	CBS 413.96	*operculata*[b]	CBS 241.83	*ramorum*	CBS 110901
cinnamomi[b] var. *parvispora*	CBS 411.96	*palmivora*[b]	CBS 236.30	*ramorum*	adc 01.01
citricola[b]	CBS 221.88	*phaseoli*[b]	CBS 556.88	*ramorum*	adc 01.06
citricola[c]	DAOM BR 681	*primulae*[b]	CBS 275.74	*ramorum*	DAOM 229466
citricola[b] (type of *P. pini*)	CBS 181.25	*pseudosyringae*	EH P96	*ramorum*	DAOM 230729
citrophthora[b]	CBS 950.87	*pseudotsugae*[b]	CBS 444.84	*ramorum*[c]	DAOM 230728
clandestina[b]	CBS 347.86	*psychrophila*[b]	CBS 803.95	*ramorum*	DAOM 230727
colocasiae[b]	CBS 955.87	*quercina*[b]	CBS 784.95	*richardiae*[b]	CBS 240.30
cryptogea[b]	CBS 113.19	*ramorum*	CBS 101327	*sinensis*[b]	CBS 557.88
cryptogea[a] f. sp. *begoniae*	CBS 468.81	*ramorum*	CBS 101326	*sojae*[b]	CBS 418.91
drechsleri[b]	CBS 291.35	*ramorum*	CBS 110538	*sojae*[b]	CBS 382.61
erythroseptica[b]	CBS 129.23	*ramorum*	CBS 110601	sp.[b] ('aquatica')	CBS 363.79
erythroseptica[b] (type of *P. himalayensis*)	CBS 357.59	*ramorum*	CBS 110543	sp.[b] ('marine')	CBS 215.85
erythroseptica[b] var. *pisi*	adc 99.69	*ramorum*	CBS 110537	*syringae*[b]	CBS 132.23
europea[b]	CBS 109049	*ramorum*	CBS 110541	*syringae*[b]	CBS 367.79
fragariae[b] v. *fragariae*	CBS 209.46	*ramorum*	CBS 110539	*tartarea*[b]	CBS 208.95
fragariae[b] var. *rubi*	CBS 967.95	*ramorum*[b]	CBS 109279	*tentaculata*[b]	CBS 552.96
gonapoyides[b]	CBS 554.67	*ramorum*	CBS 101554	*tropicalis*[b]	CBS 434.91
heveae[b]	CBS 296.29	*ramorum*	CBS 110545	*uliginosa*[b]	CBS 109054
hibernalis[b]	CBS 522.77	*ramorum*	CBS 110548	*vignae*[b]	CBS 241.73
humicola[b]	CBS 200.81	*ramorum*	CBS 101553		
idaei[b]	CBS 971.95	*ramorum*	CBS 101329		
ilicis[b]	CBS 255.93	*ramorum*	CBS 101551		
infestans[bc]	CBS 366.51	*ramorum*[b]	CBS 101332		
insolita[b]	CBS 691.79	*ramorum*	CBS 101330		
iranica[b]	CBS 374.72	*ramorum*	CBS 110546		
kandelii[b]	CBS 111.91	*ramorum*	CBS 101548		
		ramorum	CBS 101550		

[a] CBS, Centraalbureau voor Schimmelcultures; DAOM, Canadian Agriculture and Agri-Food Canada, Ottawa; adc, A. W. A. M. de Cock; ATCC, American Type Culture Collection; and EH, E. Hansen collection.

[b] Samples used in the comparison of internal transcribed spacer (ITS), β-tubulin, and elicitin TaqMan probes using real-time polymerase chain reaction (PCR) tested with 65 species of *Phytophthora.*

[c] Sample used in Figure 1: real-time PCR of *Phytophthora* samples with TaqMan, molecular beacons, and SYBR Green (β-tubulin gene).

from European and North American origin isolated from different hosts. The *Phytophthora* collection comprised isolates of 65 different species and varieties, representing most of the recognized *Phytophthora* species currently available in pure culture. Mycelium was cultivated and DNA was extracted following the procedures described in De Cock et al. (15) or Möller et al. (43).

DNA sequencing. The primers listed in Table 2 were used to amplify three gene regions of the nuclear DNA of *P. ramorum* and related *Phytophthora* species by PCR (Table 1). Primers for the β-tubulin and the elicitin genes were designed based on sequences of these genes obtained from *P. parasitica* (GenBank accession no. S67432) for elicitin, and *P. cinnamomi* (GenBank accession no. U22050) for β-tubulin. ITS primers used were from Bakkeren et al. (4) and Mazzola et al. (41), and the sequence AY038050 from GenBank for *P. ramorum* was used for alignment. These nuclear regions were selected because of the high level of sequence divergence among species that were observed in preliminary results and large number of sequence entries available in public databases.

Genomic DNA from *P. ramorum* (CBS 101553, DAOM 230728) and other species was amplified using these genus-specific primers. Most reactions yielded a single band. However, multiple bands were amplified for the elicitin gene of *P. lateralis* ATCC 201856, *P. cactorum* BR675, *P. citricola* BR 681, *P. cinnamomi* BR 680, and *P. infestans* CBS 366.51. A band of approximately 280 bp was cut and extracted from agarose gels with the QIAEX II agarose gel extraction kit (Qiagen, Valencia, CA). PCR products were then re-amplified.

PCR products were purified with the QIAquick PCR purification kit (Qiagen) using the microfuge method, quantified, and sequenced using the same primers as for PCR. Sequencing reactions were performed with a Big Dye Terminator Sequencing kit on an ABI 310 automated sequencer (PE Applied Biosystems, Foster City, CA). Both strands were sequenced with the primers listed in Table 2. Sequences were aligned using Sequencher version 4.0.5 (Gene Codes Corporation, Ann Arbor, MI) and MegAlign version 5.08 (DNASTAR Inc., Madison, WI) using Clustal W. The sequences were deposited in GenBank and the accession numbers are listed in Table 3.

Design of primers. The alignments of sequences listed in Table 3 were used to design PCR primers specific to *P. ramorum* (Table 2) using the software Primer Premier 5.00 (Premier Biosoft International, Palo Alto, CA). The selection criteria were the following: Tm (melting temperature) 55 to 65°C, primer length 18 to 22 bp, and absence of secondary structure whenever possible. Specific primers were designed so that the nucleotides unique to the target were at the 3′ end position of the primer. In primers Phy_ram_482U_LNA F and Prameli259L R (Table 2), positioning the discriminating site at the 3′ end resulted in secondary structures and the primers were moved toward the 5′ end. The primer pairs were designed such that PCR products were shorter than 200 bp, an important parameter for RT-PCR. In cases where only single nucleotide differences were present and unmodified primers did not allow specific amplifications, primers were synthesized with a lock nucleic acid (LNA) (7) (Proligo LLC, Boulder, CO) to increase specificity. The ITS primers were different from other published primers (18,26,27) and instead targeted positions 622 to 755 with mismatches at the 3′ site and the LNA modification.

Design of molecular beacon and TaqMan probes. Molecular beacons were designed using Mfold version 3.1 (DNA mfold server: 1996 to 2003, Michael Zuker, Rensselaer Polytechnic Institute) and Beacon designer 3 software (Premier Biosoft International, Palo Alto, CA) to calculate the Tm and the structure of the molecule. The molecular beacon was labeled with fluorescein (6-FAM[tm]) at the 5′ end and with the quencher Dabcyl at the 3′ end (6,55,56). TaqMan probes (28) were designed with Primer Premier 5.00. We used the following parameters for the design: Tm 10°C higher than the primers, 15 to 30 bp in length and the total number of G's or C's in the last five nucleotides at the 3′ end of the primer not exceeding two. The mismatching nucleotide was positioned as close as possible to the middle of the probe rather than at the ends while avoiding positions with secondary structures. The TaqMan probes were labeled with fluorescein (6-

TABLE 2. Primers and probes used for polymerase chain reaction assays targeting *Phytophthora* spp. and *P. ramorum*

Name[a]	Primer sequence	Region	Notes
Universal to *Phytophthora* species			
Oom-Btub-up415 F	5′-CGCATCAACGTGTACTACAA-3′	β-tubulin	≈539 bp
Oom(Ph)-Btub-lo954 R	5′-GCACACCAGGTGGTTC-3′		
Oom(Ph)-Btub-up901 F	5′-TACGACATTTGCTTCCG-3′	β-tubulin	≈500 bp
Oom-Btub-lo1401 R	5′-CGCTTGAACATCTCCTGG-3′		
UN-UP18S42 F[b]	5′-CGTAACAAGGTTTCCGTAGGTGAAC-3′	ITS	≈350 bp
OOM-LO5.8S47 R[c]	5′-ATTACGTATCGCAGTTCGCAG-3′		
OOM-UP5.8S-55 F[c]	5′-TGCGATACGTAATGCGAATT-3′	ITS	≈650 bp
UN-LO28S22 R[b]	5′-GTTTCTTTTCCTCCGCTTATTGATATG-3′		
Phy_elicitor1 F	5′-GCCCTCGTCGGCTCCAC-3′	Elicitin	≈300 bp
Phy_elicitor2 R	5′-GTGAACACGTTGAGTACCAGGC-3′		
Specific to *P. ramorum*[d]			
Phy_ram_482U_LNA F	5′-GGC**G**CTGTACGACATTTG-3′		LNA modified 171 bp
Phy_ram_653L_LNA R	5′-ACGCGGGAACGGAATCA**A**-3′	β-tubulin	
ITSPrim622U F	5′-AATGACTGGTGAACCGTAGCT**G**-3′		
ITSPrimer755L R	5′-CGAAGCCGCCAACACAA**G**-3′	ITS	LNA modified 133 bp
Prameli 102U F	5′-TTCAACCAGTGCGCGACC-3′	Elicitin	
Prameli259L R	5′-GGCACAGTCAGCTCGCAGTC-3′	Elicitin	
Molecular beacon			
Beacon4. Beta tub F	[6~FAM]CGGGCTCGGACATAGCGGCGCACACCAGCCCG[Dabcyl]	β-tubulin	157 bp
TaqMan probes			
TaqMan581L R	[6~FAM]CGTGGTGATGCCGGACATAGCG[BHQ1~Q][e]	β-tubulin	
TaqManITSGB651L R	[6~FAM]AACACCGTCGATTCAAAAGCCAAGC[BHQ1~Q]	ITS	
Taq195eli R	[6~FAM]CCGTGGACGCGCACATGAGCGAGTAC[BHQ1~Q]	Elicitin	

[a] F, forward primers; R, reverse primers.
[b] Bakkeren et al. 2000 (4).
[c] Mazzola et al. 2002 (41).
[d] Polymorphisms with the most closely related species are underlined. Bases with locked nucleic acid modification are in bold box.
[e] BHQ1~Q: Black hole quencher-Quencher.

FAM™) at the 5′ end and with the quencher Black Hole Quencher™-1 (BHQ™-1) at the 3′ end (Integrated DNA Technologies Inc., Coralville, IA).

PCR amplification. Real-time PCR was performed with a DNA Engine Opticon 2 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Fluorescent molecules (SYBR Green, dual-labeled probe [TaqMan or molecular beacons]) were included in the PCR master mix (QuantiTect, Qiagen). All reactions were performed in 25 µl volumes. The DNA concentration used in the reaction was determined with a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE) and ranged from 0.05 to 60 ng/µl for *P. ramorum* and from 5 and 110 ng/µl for other *Phytophthora* sp. Molecular beacon, TaqMan and SYBR Green real-time PCR assays were used in a preliminary experiment with the three gene regions using a subset of isolates. The real-time PCR on *P. ramorum* tested on the three gene regions were made in triplicates, and the mean and standard error were calculated for each. Subsequently, TaqMan assays were used for further studies using 48 plant tissue samples from seven hosts. *P. ramorum* CBS 101553 was used as positive control, and *P. lateralis* CBS 168.42 and no template DNA were used as negative controls. The analysis software Opticon Monitor version 2.01.10 (Bio-Rad Laboratories) was used to analyze the data (cycle range set at 1 to 21). Data were exported as cycle threshold (Ct) values and analyzed for comparisons among samples in Excel spreadsheets. Statistical analyses were performed with Excel (Microsoft Excel version 9.0.3821 SR-1, Redmond, WA). The specific reaction conditions for each of the three detection technologies tested were set up as follows.

SYBR Green. The PCR assay contained 0.4 µM of each LNA primer or regular primer (Table 2), 1× QuantiTect SYBR Green PCR Kit (Qiagen), and template DNA. PCR cycling conditions were set at 95°C for 15 min, 40 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Fluorescence was read during the extension at 72°C.

TaqMan probe. The PCR assay contained 0.4 µM of each LNA primer or regular primer depending on the region used, 0.2 µM TaqMan (dual-labeled probe) (Table 2), 1× QuantiTect Probe PCR Master Mix (Qiagen), and template DNA. PCR cycling conditions were set at 95°C for 15 min, 36 cycles at 94°C for 15 s, and 65°C for 60 s (68°C for 60 s for elicitin). Cycle number was extended to 40 to 45 for β-tubulin and ITS on environmental samples to increase sensitivity. Fluorescence was read during the extension at 65 to 68°C.

Molecular beacon. The PCR assay contained 0.4 µM of each LNA primer for β-tubulin (Table 2), 0.2 µM molecular beacon probe (Table 2), 1× QuantiTect Probe PCR Master Mix, and template DNA. PCR cycling conditions were set at 95°C for 15 min, 36 cycles at 94°C for 15 s, 65°C for 30 s, and 72°C for 30 s. Fluorescence was read after the annealing at 65°C. This allows the molecular beacon to open and anneal with the target sequence.

Standard curves. Serial dilutions of *P. ramorum* DNA were used to calculate amplification efficiency with TaqMan assays. DNA serial dilutions consisted of *P. ramorum* strain CBS 101327 with an estimated initial concentration of 10 ng/µl diluted in a series of 1:10 from 1 ng/µl to 1 fg/µl. Three dilution series were done with Tris buffer, 10 mM, pH 8.0, *Rhododendron* sp. DNA (38.8 ng/µl) and *P. monticola* DNA (3.5 ng/µl). Standard curves were replicated for each marker twice for each dilution. The results were analyzed by plotting the Log of template concentration against Ct values. PCR efficiency was calculated with the formula $E = (10^{(-1/\text{slope})} - 1) \times 100$, where E is the amplification efficiency and the slope is derived from the plot of Log of template concentration versus Ct. A slope of 3.32 translates into 100% efficiency of amplification.

DNA extraction and pathogen isolation from infected plant material. To test sensitivity and specificity of the assays on infected plant material, environmental samples were obtained from different hosts either healthy or naturally infected by the pathogen. Samples were collected on 14 June 2005, at Pfeiffer Big Sur State Park, Monterey County, CA, and processed at the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) station in Salinas, CA. Protocols for sample collection and handling followed California Oak Mortality Task Force (Pest alert 6, Diagnosis and Monitoring of Sudden Oak Death, March 2002) recommendations. Briefly, symptoms resembling SOD were sought in the field. Leaf blights and diseased twigs of plants belonging to host species such as bay laurel (*Umbellularia californica*), madrone (*Arbutus menziesii*), coffee berry (*Frangula californica*), Christmas berry (*Heteromeles arbutifolia*), and tan oak (*Lithocarpus densiflorus*) were individually collected, annotated, and stored in cold for transportation and processing. For comparison purposes samples of healthy hosts such as camellia (*Camellia* spp.) and live oak (*Q. agrifolia*) were collected at the USDA/ARS station in Salinas, CA. Samples were superficially cleaned from excess soil and debris with sterile water and blot dried. For species in which the pathogen typically causes cankers such as tan oaks and live oaks, often necrosis of twigs and wilting of small branches signal ongoing infections. Therefore, instead of sampling the bark, wilting twigs were collected. This type of sample has been shown to correlate with *P. ramorum* infection and to subsequently produce symptoms such as shoot tip dieback, leaf flagging, or the formation of a Shepard's crook (14). Once in the laboratory, tan oak leaves including the petioles were used as starting material to isolate the pathogen. Particular care was given to use adjacent tissues of the infected sample to perform the in vitro culture and DNA isolation of the pathogen.

TABLE 3. Isolates of *Phytophthora* species sequenced in this study and GenBank accession numbers for β-tubulin and elicitin genes

Species	Collection number[b]	GenBank accession number[a]	
		β-Tubulin	Elicitin
P. ramorum	DAOM 229466	AY766201	–
P. ramorum	DAOM 230729	AY766202	–
P. ramorum	DAOM 230728	AY766203	AY766222
P. ramorum	DAOM 230727	AY766204	–
P. ramorum	CBS 109279	AY766205	–
P. ramorum	CBS 109278	AY766206	–
P. ramorum	CBS 101554	AY766207	–
P. ramorum	CBS 101551	AY766208	–
P. ramorum	CBS 101550	AY766209	–
P. ramorum	CBS 101548	AY766210	–
P. ramorum	CBS 101332	AY766211	–
P. ramorum	CBS 101330	AY766212	–
P. ramorum	CBS 101327	AY766213	–
P. ramorum	CBS 101552	AY766214	–
P. ramorum	CBS 101326	AY766215	–
P. ramorum	adc 01.01	AY766216	–
P. ramorum	adc 01.06	AY766217	–
P. ramorum	CBS 101553	AY766218	AY766223
P. ramorum	CBS 110537	–	AY766231
P. ramorum	CBS 110541	–	AY766232
P. ramorum	CBS 110545	–	AY766233
P. ramorum	CBS 110900	–	AY766234
P. ramorum	CBS 110901	–	AY766235
P. lateralis	CBS 168.42	AY766219	AY766225
P. cactorum	CBS 108.09	AY766220	–
P. cinnamomi	CBS 144.22	AY766221	–
P. lateralis	ATCC 201856	–	AY766224
P. cactorum	DAOM BR 675	–	AY766226
P. cinnamomi	DAOM BR 680	–	AY766227
P. infestans	CBS 366.51	–	AY766228
P. citricola	DAOM BR 681	–	AY766229

[a] Alignments are available for those sequences as a popset on Genbank. – Indicates no sequence data.

[b] CBS, Centraalbureau voor Schimmelcultures; DAOM, Canadian Agriculture and Agri-Food Canada, Ottawa; adc, A. W. A. M. de Cock; and ATCC, American Type Culture Collection.

Host DNA was isolated using the USDA-APHIS protocol with the Qiagen DNeasy kit (1). For control purposes, most of the healthy samples of live oak and camellia were spiked with *P. ramorum* CBS 101553 and *P. pseudosyringae* EH P96 DNA. Aliquots of the DNA samples were coded and sent blind to our laboratory for testing. Pathogen isolation was done on PARP (pimaricin-ampicillin-rifampicin-PCNB agar) media (31).

In addition, environmental samples were tested with a *cox*I/II TaqMan in RT-PCR (54). To confirm that *P. ramorum* was present in PARP (–)/RT-PCR (+) amplicons, *Phytophthora* genus-specific primers were used to amplify the spacer regions between the *cox*I and II genes and this amplicon was sequenced as previously described (39).

RESULTS

***Phytophthora* sequence divergence.** Approximately 2 kb for the two nuclear genes and ITS ribosomal DNA region was sequenced from several *Phytophthora* species (Table 3). Depending on the species, close to 866, 260, and 780 bp were sequenced for β-tubulin, elicitin, and the ITS, respectively. Divergence was the highest for β-tubulin and elicitin for comparisons between the two closely related species *P. ramorum* and *P. lateralis*. A total of 18/866 bases for β-tubulin (2.2% divergence) and 4/93 bases for elicitin (4.5% divergence) were polymorphic between these two species. In contrast, the ITS contained only 13 polymorphisms out of 783 bases (1.4% divergence) between these two species. However, divergence among species was greater for comparisons with more distant *Phytophthora* species. For example, divergence between *P. ramorum* and *P. cactorum* was 6.0, 11.4, and 10.8% for β-tubulin, elicitin, and ITS, respectively. Similarly, divergence between *P. ramorum* and *P. cinnamomi* was 7.5, 9.8, and 12.2% for these three genes. A similar level of sequence divergence was observed in comparisons with *P. citricola*, *P. infestans*, and *P. nicotianae*. However, the low number of polymorphisms in β-tubulin was still sufficiently high among the distant species to design specific primers and probes for *P. ramorum*.

β-Tubulin PCR assays with TaqMan, molecular beacon, and SYBR Green. Using the β-tubulin primers, the three PCR reporter technologies were compared. PCR primers specific to *P. ramorum* were designed for β-tubulin to amplify an amplicon of 171 bp. The primers contained several nucleotides polymorphic among *Phytophthora* species used in this study (Tables 2 and 3). However, because of the low divergence between *P. ramorum* and *P. lateralis*, and the constraints with designing primers to amplify amplicons less than 200 bp for real-time PCR assays, both β-tubulin primers contained only one polymorphic nucleotide and were modified with LNA to increase specificity. Specificity of the β-tubulin assays was compared for TaqMan, molecular beacon, and SYBR Green in real-time PCR initially on *P. ramorum*, *P. lateralis*, *P. cactorum*, *P. cinnamomi*, *P. citricola*, and *P. infestans* (Fig. 1), representing closely related as well as more diverged species.

All real-time PCR assays targeting the β-tubulin gene yielded Ct values for the *P. ramorum* samples, but the Ct varied according to the reporter technology used. The TaqMan, SYBR Green, and molecular beacon assays yielded Ct values of 22.04, 22.77, and 25.08, respectively. No amplifications were observed with the nontarget *Phytophthora* species tested. Since PCR efficiencies were comparable among the three assays (data not shown) and based on the fact that TaqMan probes are easier to design than molecular beacon, we designed TaqMan reporter probe assays for the comparison of PCR efficiencies in the three genes targeted (Table 2).

Comparison of ITS, β-tubulin, and elicitin TaqMan probes using real-time PCR. To compare the specificity and sensitivity

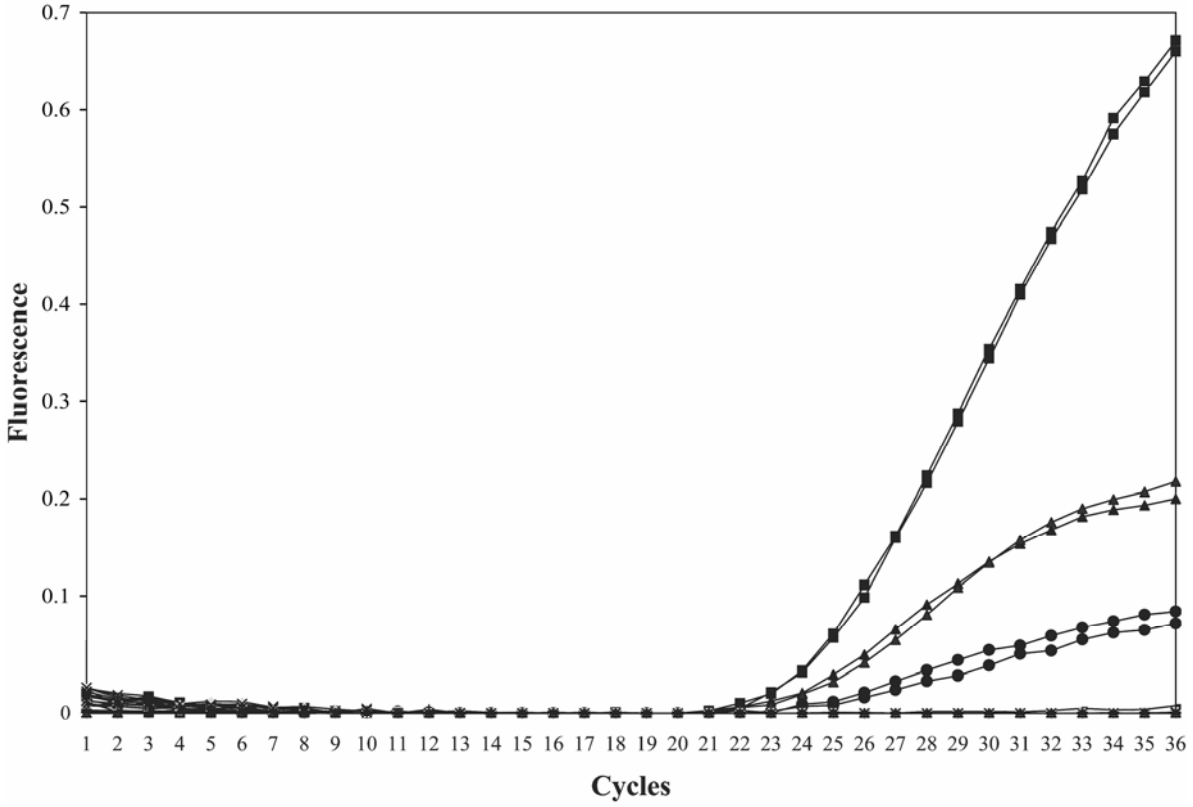

Fig. 1. Real-time polymerase chain reaction of *Phytophthora* species assayed with TaqMan (■), molecular beacons (●), and SYBR Green (▲), targeting the β-tubulin gene. The *Phytophthora* species used were: *P. ramorum* 230928, *P. ramorum* 230991; all other species used are *P. lateralis* ATCC 201856, *P. cactorum* BR675, *P. cinnamomi* BR680, *P. citricola* BR681, *P. infestans* M0014, and a negative control (water) and are represented by the ×. Threshold was set at 0.009, with a cycle range of 1 to 15.

of probes in different gene regions, we focused on the TaqMan assay using DNA from 65 species of *Phytophthora* (Table 1) from pure cultures. Again, the Ct values for *P. ramorum* varied according to the gene region targeted. Ct values were 20.8 for the ITS-TaqMan and 21.4 for the elicitin-TaqMan real-time PCR assays. The β-tubulin TaqMan assay yielded the highest Ct values (Ct = 24.4). None of the remaining *Phytophthora* spp. templates yielded Ct values with any of the TaqMan real-time PCR assays, indicating that the assays were highly specific, including species that frequently cross-react with *P. ramorum* such as *P. pseudosyringae*, *P. nemorosa*, and *P. kernoviae* (not shown).

Standard curves with serial dilutions of *P. ramorum* DNA were generated to calculate amplification efficiency. Amplification efficiencies were 100, 91, and 100%, in TaqMan assays targeting ITS, elicitin, and β-tubulin diluted in Tris-HCl (Fig. 2). Addition of plant DNA from pine changed amplification efficiencies for these markers to 86, 100, and 91%, respectively, while rhododendron DNA changed them to 78, 100, and 91% (data not shown). The limit of detection was 1, 10, and 100 fg for the ITS, elicitin, and β-tubulin TaqMan assays, respectively (Fig. 2).

To determine whether the developed assays were applicable to a broad range of *P. ramorum* sources, 30 isolates of *P. ramorum* from Europe and North America (Table 4) were tested with the TaqMan probes for the three different gene regions. All reactions with *P. ramorum* isolates resulted in fluorescent curves that rose above the threshold value, regardless of the gene targeted by the real-time PCR assay (Table 4). Again, the Ct values varied according to the gene targeted, with Ct values for *P. ramorum* averaging 20.02 (standard error [SE] 2.97), 20.65 (SE 2.46), and 24.70 (SE 2.57) for the ITS, elicitin, and β-tubulin, respectively (Table 4). The relatively large SE reflects the variability in initial DNA concentration (ranging from 2 to 32 ng).

Detection of *P. ramorum* in infected samples. To determine the reliability of the assays to amplify *P. ramorum* DNA from infected plant samples and from samples spiked with *Phytophthora* DNA, the three TaqMan real-time PCR assays were used to test 48 different plant extracts from seven hosts in a blind test (Table 5). All samples were plated on PARP-V8 medium and tested using *cox*I/II RT-PCR (54) at the USDA-ARS laboratory in Salinas, CA, and assayed in a blind test at the Laurentian Forestry Centre laboratory. All samples spiked with *P. ramorum* were detected accurately using all of the assays developed in this study, and were concordant with the *cox*I/II assays. All of the environmental samples gave concordant results for the four molecular assays, including 63% negatives and 13% positives (excluding spiked samples). All environmental samples that were naturally infected and yielded *P. ramorum* cultures on PARP-V8 as well as positive PCR results with the *cox*I/II assay were positive with the ITS, β-tubulin, and elicitin assays. There was some level of discordance among the results for the molecular assays and the results of the culture isolation; the pathogen was not recovered from 24% of the samples that were PCR positive. The presence of *P. ramorum* in all but four of these samples was confirmed by sequence analysis of the spacer region between the *cox*I and II genes amplified using *Phytophthora* genus-specific primers (data not shown). The sample spiked with *P. pseudosyringae* was negative with all molecular assays. There were some cross reactions with *P. lateralis* at a high DNA concentration (8 ng/μl) with the elicitin TaqMan assay with a Ct value that was higher than the target. However, the results of the ITS assay were negative and the Ct for the β-tubulin assay was 41, above what normally would be considered a positive sample.

Ct values in samples from infected material were higher than in the pure culture assays described in the previous sections. The Ct values ranged from 23 to 39 (TaqMan-ITS) to 28 to 40 (TaqMan-β-tubulin) and 21 to 34 (TaqMan-elicitin) in environmental samples that were tested positive (Table 5).

DISCUSSION

Real-time PCR has been used in plant pathology for DNA quantification and diagnosis of several plant pathogens (52). The

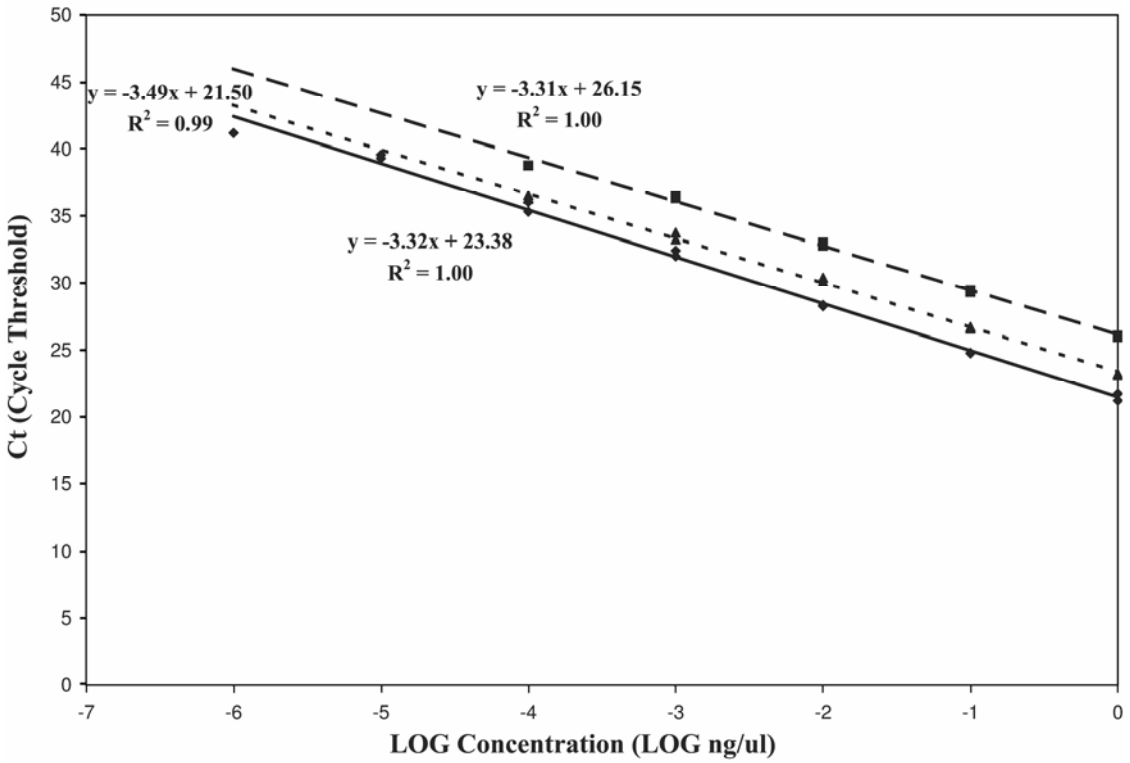

Fig. 2. Standard curves based on dilution of *Phytophthora ramorum* DNA for TaqMan assays targeting internal transcribed spacer (ITS), β-tubulin, and elicitin. Plots represent cycle threshold (Ct) versus the Log of DNA concentration. *P. ramorum* DNA was diluted from concentrations of 1 ng/μl to 1 fg/μl in Tris buffer, pH 8.0, 10 mM. Assays were as follows: ■ β-tubulin TaqMan, ▲ elicitin TaqMan, and ♦ ITS TaqMan.

potential of molecular detection in plant pathology has been shown in several studies (40,42,52). The advantages include speed of the assays and, the possibility of detecting nonculturable pathogens with high sensitivity. The disadvantages include cost, the need for well-equipped laboratories, and the inability to determine if the target organism is viable. Also, the risk of obtaining false positives and negatives can never be fully discounted due to the inability of fully assessing the biodiversity of target species and close relatives. To reduce the risk of incorrect identification (e.g., in regulatory applications), confirmation of morphometric properties (from culture isolation) should be included in the diagnostic protocol.

The real-time PCR assays described in this research resulted in the specific and sensitive detection of *P. ramorum* from cultures and plant samples. Tests with pure cultures of *Phytophthora* species and *P. ramorum* from a worldwide collection demonstrated the specificity of the ITS, β-tubulin, and elicitin TaqMan assays. No false positive or false negative results were observed with any of the assays consisting of dual probes and specific PCR primers, except for *P. lateralis* at high DNA concentration in the environmental samples experiment. However, Ct values were high even at those high DNA concentrations (41 for β-tubulin and 33 to 34 for elicitin) and values over 40 would be considered negative in an operational assay. In addition, the assays were tested using infected plant material from which the pathogen had been isolated, or with plant material spiked with *P. ramorum* DNA. This validation step was done as a blind test and confirmed the concordance between the various molecular assays and the increased confidence afforded by the gene region redundancy in the assays.

Our study compared molecular beacon, TaqMan, and SYBR Green assays for more than one gene region against a single target microorganism. The use of multiple gene targets increases reliability and confidence in the assays. Although it should be possible to directly multiplex the internal probe assays into single reactions, we preferred to conduct assays in separate reactions for the different gene targets. Multiplexing generally results in some competition among the PCR products and could reduce the amplification efficiency and thus the sensitivity of the assays. Molecular beacons and TaqMan probes also have built-in redundancy of polymorphisms and give two potential levels of specificity in their design: the PCR primers and the internal dual-labeled probe. In the assays we developed, this provided several polymorphic nucleotides between the target *P. ramorum* and related taxa. Even for the closely related *P. lateralis*, which has only 1.4% divergence with *P. ramorum* in the ITS region, it was possible to design a TaqMan assay with one polymorphic nucleotide per PCR primer, and an additional three polymorphic nucleotides within the TaqMan probe. The extra specificity given by the internal probes could be significant in reducing the probability of false positives. As SYBR Green intercalates nonspecifically into all double-stranded DNA molecules during the reaction, the assay must rely entirely on the PCR primers for specificity (21). In addition, the inhibition of the Taq DNA polymerase by SYBR Green could result in lower specificity (2). In our experiments, the annealing temperature was lowered in the reactions using SYBR Green compared with those using internal probes to counter this inhibition. Since the same primers were used in the SYBR Green and the TaqMan and molecular beacon as-

TABLE 4. Number of cycles before fluorescence is detected (Ct) in *Phytophthora ramorum* isolates tested by real-time polymerase chain reaction using three different gene regions (internal transcribed spacer [ITS], β-tubulin, and elicitin) with TaqMan

			Ct[a]					
			ITS		Elicitin		β-Tubulin	
CBS number	Origin	Isolate number	Mean Ct[b]	Standard error	Mean Ct[b]	Standard error	Mean Ct[b]	Standard error
101327	Netherlands	PD 93/56	17.71	0.58	19.05	0.04	23.41	0.57
101326	Netherlands	PD 98/8/6933	19.61	0.69	20.54	0.04	24.90	0.72
110538	California	Pr65	19.26	1.61	19.82	0.24	23.76	0.62
110601	California	Pr84-sz	26.57	0.89	26.04	0.31	30.14	0.79
110543	Oregon	Pr159	17.23	0.17	19.17	0.35	23.07	0.71
110537	California	Pr52	18.92	0.51	21.29	0.23	24.75	0.56
110541	California	Pr86	21.10	0.26	21.36	0.37	24.87	0.67
110539	California	Pr70	18.27	0.68	18.86	1.50	23.35	0.19
109279	Germany	BBA 13/99-1	18.80	0.55	19.29	0.18	23.98	0.49
101554	Germany	BBA 2/4	19.52	0.94	19.84	0.20	24.21	1.19
110545	Poland	Rh/2/00	19.67	1.02	19.80	0.44	23.83	0.97
110548	France	adc 02.09	27.86	0.74	27.36	0.42	31.61	0.60
101553	Germany	BBA 9/95 Type	20.66	0.51	21.05	0.17	25.18	0.61
101329	Netherlands	PD 98/8/6285	16.91	0.57	18.71	0.24	22.73	0.48
101551	Germany	BBA 12/98	19.39	0.42	19.38	0.38	23.66	0.72
101332	Netherlands	PD 94/844	17.78	0.86	18.48	0.26	22.56	0.72
101330	Netherlands	PD 98/8/5233	18.65	0.62	19.63	0.19	24.17	0.76
110546	Poland	Rh/6/00	27.71	1.20	26.61	0.32	31.37	1.22
101548	Germany	BBA 69082	18.44	1.24	19.00	0.33	22.74	1.06
101550	Germany	BBA 14/98-a	19.93	0.89	20.87	0.24	24.72	0.83
110544	California	PrPRJL3.5.3	18.19	1.17	20.13	0.29	23.72	0.87
109278	Germany	BBA 16/99	19.67	0.70	20.40	0.39	24.59	0.61
101552	Germany	BBA 9/3	18.97	0.61	17.94	0.15	22.04	0.60
101549	Germany	BBA 104/5	19.80	1.05	19.81	0.19	24.31	0.88
101331	Netherlands	PD 98/8/2627	17.55	0.70	18.44	0.15	22.90	0.84
110535	California	Pr03	19.40	0.51	19.92	0.06	23.07	0.67
110547	Poland	Rh/122/98	26.06	1.01	25.67	0.11	30.20	0.88
110536	California	Pr04	19.04	0.75	20.75	0.76	23.58	0.79
110542	California	Pr110	19.47	0.40	20.47	0.48	24.12	0.77
110534	California	Pr01	18.39	0.91	19.84	0.46	23.39	0.70
Average Ct by TaqMan			20.02	2.97	20.65	2.460	24.70	2.57

[a] Ct, number of cycles before fluorescence threshold is reached. The threshold was never reached for *P. lateralis* and 64 other *Phytophthora* species (data not shown).

[b] Mean Ct was calculated with triplicates and standard error was obtained.

says, this could have resulted in a lower specificity in SYBR Green.

The TaqMan assays developed here produced very high levels of amplification efficiency. The fluorescence continued to increase even after 40 cycles in some trials, suggesting the reaction components had not yet become limiting even if the reaction was very efficient. In a dilution series of *P. ramorum* DNA from 1 fg to 10 ng, the TaqMan system with elicitin, β-tubulin, and ITS required on average 3.30, 3.30, and 3.60 cycles per 10-fold dilution to pass the threshold, which is very close to the theoretical value of 3.33

TABLE 5. Detection of *Phytophthora ramorum* in infected plant material by real-time polymerase chain reaction using three gene regions with TaqMan and comparison with other methods

Samples	Host[b]	PARP-V8 isolation	TaqMan *cox*I/II[c]	Ct[a] TaqMan β-Tubulin	Ct[a] TaqMan ITS	Ct[a] TaqMan Elicitin
PU2	Bay laurel*	Negative	Negative	39.58	None	None
PU3	Bay laurel*	Negative	Negative	None	None	None
PU4	Bay laurel*	Negative	Negative	None	None	None
PU5	Bay laurel*	Negative	Negative	None	None	None
PU6	Bay laurel*	Negative	Negative	None	None	None
PU8	Tan oak*	Negative	Negative	None	None	None
PU9	Tan oak*	Negative	Negative	None	None	None
PU10	Tan oak*	Negative	Negative	None	None	None
PU11	Tan oak*	Negative	Negative	None	None	None
PU12	California coffee berry*	Negative	Negative	None	None	None
PU13	California coffee berry*	Negative	Negative	None	None	None
PU14	California coffee berry*	Negative	Negative	None	None	None
PU15	Bay laurel*	Negative	Negative	None	None	None
PU21	Bay laurel*	Negative	Negative	None	None	None
PU25	Bay laurel*	Negative	Negative	None	None	None
PU26	Bay laurel*	Negative	Negative	None	None	None
PU27	Bay laurel*	Negative	Negative	None	None	None
PU29	Bay laurel*	Negative	Negative	None	None	None
PU30	Tan oak*	Negative	Negative	None	None	None
PU31	Tan oak*	Negative	Negative	None	None	None
PU33	Tan oak*	Negative	Negative	None	None	None
PU36	Tan oak*	Negative	Negative	None	None	None
PU38	Tan oak*	Negative	Negative	None	None	None
PU39	Christmas berry*	Negative	Negative	None	None	None
PU18	Bay laurel*	**Positive**	**Positive**	**36.04**	**32.83**	**30.39**
PU19	Bay laurel*	**Positive**	**Positive**	**38.47**	**36.59**	**33.69**
PU20	Bay laurel*	**Positive**	**Positive**	**36.57**	**33.37**	**30.29**
PU28	Bay laurel*	**Positive**	**Positive**	**38.37**	**33.56**	**30.66**
PU32	Tan oak*	**Positive**	**Positive**	**34.10**	**29.57**	**29.00**
PU1	Madrone*	Negative	**Positive**	**28.45**	**23.59**	**22.70**
PU16	Bay laurel*	Negative	**Positive**	**None**	**39.21**	**33.76**
PU17	Bay laurel*	Negative	**Positive**	**37.43**	**36.01**	**31.54**
PU22	Bay laurel*	Negative	**Positive**	**39.56**	**39.52**	**34.45**
PU23	Bay laurel*	Negative	**Positive**	**38.80**	**34.68**	**32.27**
PU24	Bay laurel*	Negative	**Positive**	**36.27**	**28.26**	**30.57**
PU34	Tan oak*	Negative	**Positive**	**40.61**	**38.48**	**31.81**
PU35	Tan oak*	Negative	**Positive**	**39.96**	**39.64**	**31.28**
PU37	Tan oak*	Negative	**Positive**	**40.55**	**37.50**	**32.09**
Controls and spiked samples						
PU40	Live oak**	**Spiked with 1.3 ng/µl**[d]	**Correct**	**28.29**	**22.77**	**21.40**
PU41	Live oak**	**Spiked with 0.65 ng/µl**[d]	**Correct**	**30.33**	**24.11**	**22.48**
PU44	Live oak**	**Spiked with 121 pg/µl**[d]	**Correct**	**33.52**	**27.64**	**25.64**
PU43	Live oak**	**Spiked with 62 pg/µl**[d]	**Correct**	**32.24**	**24.56**	**25.35**
PU42	Live oak**	Spiked with 4.3 ng/µl[e]	Correct	None	None	None
PU45	No plant DNA**	***P. ramorum* CBS 101553**	**Correct**	**25.88**	**19.33**	**19.10**
PU46	Camellia**	**Spiked with 3.75 ng/µl**[d]	**Correct**	**33.85**	**20.25**	**19.12**
PU47	Camellia**	**Spiked with 125 pg/µl**[d]	**Correct**	**32.28**	**28.24**	**25.90**
PU48	Camellia**	Not spiked	Correct	None	None	None
PU49	Camellia**	Not spiked	Correct	None	None	None
PC1	Positive control, *P. ramorum* CBS 101553			**29.05**	**25.13**	**22.27**
Plat	*P. lateralis* CBS 168.42			>41.00	None	>33.00
NC	Negative control, no template DNA			None	None	None

[a] Threshold was set at 0.009 in the Opticon Monitor software.

[b] Host scientific names: bay laurel, *Umbellularia californica*; California coffee berry, *Rhamnus californica*; camellia, *Camellia japonica*; Christmas berry, *Heteromeles arbutifolia*; live oak, *Quercus agrifolia*; madrone, *Abutus menziesii*; and tan oak, *Lithocarpus densiflorus.* *, sample origin: Monterey County, CA, Big Sur area; **, sample origin: Monterrey County, CA, USDA-ARS Salinas station.

[c] Bold positives samples, confirmed by sequence analysis of the spacer region between the *cox*I and *cox*II genes.

[d] Spiked with *P. ramorum* CBS 101553; regression equations derived from plotting Ct versus Log(concentration) in spiked samples: β-tubulin, $y = -3.46x + 29.64$, $r^2 = 0.90$; ITS, $y = -3.60x + 28.82$, $r^2 = 0.96$; and elicitin, $y = -3.41x + 21.71$, $r^2 = 0.98$.

[e] Spiked with *P. pseudosyringae* EH P96.

if the DNA molecules are doubled at every cycle (Fig. 2). The lower than 100% amplification efficiency could be explained by several factors, including the addition of plant DNA in our dilution series to reproduce amplification conditions encountered when processing field samples. In contrast, SYBR Green and molecular beacon fluorescence reached a plateau probably because fluorescence is generated "de novo" at every cycle by the intercalation of the SYBR Green dye in the double-stranded DNA during extension or by the hybridization of the molecular beacon at annealing. Alternatively, it is possible that the dye used for the TaqMan probe was more fluorescent than in the other assays, although FAM was used in all those assays and different quenchers were used for molecular beacon and TaqMan. However, the Ct values and the overall intensity of fluorescence of each sample followed the same trends among the three types of DNA detection assays (Fig. 1).

DNA extracts from 65 of the 84 documented *Phytophthora* species were tested in our study with the ITS, β-tubulin, and elicitin TaqMan assays. These *P. ramorum*-specific assays did not cross react with other *Phytophthora* species when tested at 5 to 110 ng, including the closely related *P. hibernalis*. However, at high concentrations (8 ng/µl), we did get some cross-reaction with *P. lateralis* using the *P. ramorum*-specific assays for the elicitin marker. Similar cross-reactivity has been reported between *P. ramorum* and *P. lateralis* for an ITS TaqMan assay (53). The three TaqMan assays also correctly detected *P. ramorum* in the plant tissue extracts tested. The sensitivity of the TaqMan assay was approximately four to seven cycles higher when used with the ITS and elicitin probes compared with the mean Ct of β-tubulin with the same samples (Table 5). This would make the TaqMan-ITS and TaqMan-elicitin assays approximately 1 or 2 orders of magnitude more sensitive than the β-tubulin assay with any of the techniques used.

By comparing standard curves, we could determine that the ITS TaqMan assay was the most sensitive, followed by the elicitin and β-tubulin assays. The TaqMan ITS assay yielded Ct values that were on average 4.5 cycles lower than the β-tubulin assays and 1.94 cycles lower than the elicitin assay. This is somewhat expected since rDNA consists of a tandemly repeated gene cluster, and therefore a single cell contains several copies of the cistron (61). Elicitin is also known to have multiples copies (49). By contrast, β-tubulin is usually found in fewer copies (3,57). Using genes in multiple copies allows detection of smaller amounts of target DNA compared with assays based on single copy genes. Molecular diagnosis of *P. ramorum* based on nested PCR amplification of the ITS has been reported (18,19,26). Although nested PCR can be very powerful and sensitive, it requires additional manipulations of the amplified DNA and can therefore result in higher risks of contamination, and therefore higher rates of false positives (47). Our assay also uses the ITS region but with a single round of PCR in a closed tube assay, reducing the possibility of false positives due to carryover contamination. However, direct comparison of the nested PCR protocol with the assays described here would be necessary to determine the relative merits of each test for specificity and sensitivity.

Other PCR-based methods have been used to characterize *P. ramorum* at the DNA level. Amplified fragment length polymorphism assays have been used for molecular characterization of *P. ramorum* isolates. These studies revealed that the European and North American strains of *P. ramorum* differ (8,29,30,35,50,59) and show variation. Our sequence analysis also revealed some single nucleotide polymorphism (SNP) differences between the European and North American isolates in the β-tubulin gene. Thus far, this polymorphism correlates completely with the origin of the strains (e.g., European versus North American). We are currently developing an SNP genotyping assay that allows high throughput characterization for geographic origin (5) and is in concordance with microsatellite analysis (29).

All field samples that yielded cultures of *P. ramorum* gave positive PCR results but clearly, the PCR assays also detected *P. ramorum* consistently in some samples that *P. ramorum* could not be recovered. In all but four cases, however, sequence analysis of the spacer region between the *cox*I and II genes confirmed the presence of the pathogen. Sequencing of this spacer region has been proven to be a valuable tool for organism identification in *Phytophthora* species (38) and prior evaluations with a larger number of field samples confirmed the ability of the *cox*I/II marker system to identify the pathogen when it could not be cultured (39,54). For these four samples, clean sequence data were not obtained, perhaps due to the presence of multiple *Phytophthora* species in the sample. The high Ct values obtained when using the markers developed in this submission indicated that the amount of *P. ramorum* was probably very low. The greater sensitivity of molecular assays compared with culture assays is not entirely unexpected as culture methods can be affected by the presence of contaminants, competitors, or inhibitors (e.g., fungicides) in the tissues (23,24), as well as seasonal variation (27). Moreover, nonviable pathogen propagules would result in positive PCR assays, but cultures would not be obtained.

The real-time PCR assays developed here can be a useful tool to detect *P. ramorum* rapidly and sensitively from pure cultures and plant tissue samples. Our approach allows for redundancy both in the gene regions targeted by designing assays that take advantage of SNPs at priming sites as well as at internal sites for probes. This approach, combined with use of assays for multiple gene/spacers targets, should greatly increase the confidence level of these assays and reduce the potential for false positive results in molecular testing.

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