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Detection of mRNA by reverse-transcription PCR as an indicator of viability in *Phytophthora ramorum*

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Summary

In the last few decades, the use of molecular tools has greatly improved the efficiency of plant disease diagnosis. However, one of the major setbacks of most molecular diagnostic approaches is their inability to differentiate between dead and viable pathogens. We propose a new strategy for the detection of plant pathogens, based on the use of mRNA as a viability marker, on the basis that mRNA degradation in dead cells is significantly more rapid than that of DNA. A real-time reverse-transcription PCR (RT-PCR) assay targeting the mRNA of the subunit I of the *cytochrome oxidase* gene was designed for *Phytophthora ramorum*, the causal agent of sudden oak death and ramorum blight. In controlled laboratory tests, the developed RT-PCR assay did not detect the target mRNA a week after the pathogen had been killed by rapid lyophilization, while DNA of the pathogen could still be detected 3 months after the pathogen had died. The RT-PCR assay was then compared with a traditional culturing approach using PARP selective medium and two nested real-time PCR techniques on symptomatic California bay laurel leaves. Samples were either collected in three different sites in July, or in the same site but in three different seasons. Overall, RT-PCR results showed less positive samples than DNA-based nested PCR techniques ($p < 0.0001$), but more than culturing ($p = 0.017$). Nested PCR-positive but RT-PCR-negative samples may not be viable. On the other hand, RT-PCR-positive but culture-negative samples may be viable but dormant. A comparative analysis of the results indicated that RT-PCR and culturing provide comparable results when climatic conditions are optimal for the pathogen, but RT-PCR may be the most accurate approach to determine pathogen viability when climatic conditions are less than optimal for the pathogen.

1 Introduction

Introductions of exotic pathogens and pests in new areas have significant ecological, economic and evolutionary consequences and may irreversibly alter the structure and function of ecosystems (Castello et al. 1995; Enserink 1999; Everett 2000). *Phytophthora ramorum* (Werres et al. 2001), causal agent of sudden oak death (SOD), is a recent example of an introduction of a pathogen (Ivors et al. 2006; Mascheretti et al. 2008) that has resulted in a catastrophic epidemic of forest trees in California (Rizzo et al. 2005).

Phytophthora ramorum is a polyphagous pathogen capable of infecting over 100 plant hosts both in wildlands and in commercial nurseries; this number is deemed to increase because our understanding of its host range is still imperfect. In California's natural ecosystems, tanoaks (*Notholithocarpus densiflorus*) and several species of red oaks (*Quercus* spp.) can be locally decimated by the SOD pathogen, while it is other plant species such as bay laurels (*Umbellularia californica*) that are mostly responsible for the spread of the disease (Davidson et al. 2005, Davidson et al. 2008). *P. ramorum* also infects many ornamental plants, such as camellia and rhododendron, causing a syndrome named 'ramorum blight' characterized by symptoms, such as leaf spots, twig blight and shoot dieback (Davidson et al. 2003). Other less virulent *Phytophthora* species, including *P. pseudosyringae* and *P. nemorosa,* are often associated with ramorum blight. These two species have an overlapping host and geographic range with *P. ramorum* in California and Oregon (Hansen et al. 2003; Wickland et al. 2008). Given the wide host range of *P. ramorum* and its potential for spreading to new areas of the United States through nursery stocks (Goss et al. 2009), quarantine regulations, at both federal and state level, have been developed by the Animal and Plant Health Inspection Service of the U.S. Department of Agriculture (USDA) on all known host species for *P. ramorum* (Rizzo and Garbelotto 2003; Anonymous, 2007). In addition, Canada, South Korea and the European Union have placed restrictions on importation of reported *P. ramorum* host species (Schrader and Unger 2003; Garbelotto and Rizzo 2005; Anonymous 2006, 2007).

The development of accurate and rapid diagnostic methods for *P. ramorum* is crucial for the implementation of trading regulations. Because of the presence of other *Phytophthora* spp. causing similar symptoms and having comparable morphology, symptoms alone are not adequate for an accurate diagnosis of this pathogen (Hansen et al. 2003; Martin and Tooley 2003). Furthermore, successful culturing of *P. ramorum* seems dependent on aspects, including environmental conditions and host substrate (Hayden et al. 2004; Vettraino et al. 2009). To improve the efficiency of detection of this pathogen, the USDA has recently deemed DNA-based diagnosis necessary to determine whether regulated plants or plant parts are indeed free of the pathogen (Anonymous, 2007).

Real-time PCR technologies offer increasing opportunities to detect and study phytopathogenic fungi. They combine the sensitivity of conventional PCR with the generation of a specific fluorescent signal providing both real-time analysis of the reaction kinetics and quantification of specific DNA targets (Schmittgen 2001). Real-time PCR has been used to quantify the amount of *Phytophthora* DNA in several studies, and gene probe methods of detection and identification have proved to be

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inherently more specific and sensitive than traditional methods (Martin et al. 2000, 2004, 2009; Winton and Hansen 2001; Hayden et al. 2004, 2006; Kong et al. 2004; Tomlinson et al. 2005; Hughes et al. 2006, Schena and Cooke 2006a; Schena et al. 2006b; Tooley et al. 2006; Kox et al. 2007). However, one disadvantage of DNA-based methods is that they do not distinguish between living and dead organisms. Because both traditional and real-time PCR assays detect nucleic acids rather than living cells, there is a risk that nucleic acids relinquished from dead and unviable cells may lead to positive PCR signals (Scheu et al. 1998). Several studies have shown extended persistence of PCR-detectable DNA in samples after death of target cells (Josephson et al. 1993; Allmann et al. 1995). Quantitative studies of DNA degradation kinetics determined by real-time PCR have shown that the rate of degradation of DNA after cell death is variable, according to DNA binding potential of the substrate (Wolffs et al. 2005). Such variability may be justified in part by the fact that the microenvironment in which DNA is encased has different levels of accessibility for macromolecules, compared to free nucleic acids (Kloos et al. 1994).

To deal with the risk of false-positive PCR results (i.e. samples containing unviable DNA of a pathogen), many researchers have investigated the use of mRNA as a viability marker on the basis of its rapid degradation compared to that of DNA (Alifano et al. 1994; Mendum et al. 1998; Uyttendaele et al. 1999; Vettraino et al. 2010). Detection of mRNA might be a good indicator of living or of recently dead cells because mRNA is turned over rapidly in living cells, with most species having mRNA with half-life of only a few minutes, and degrading rapidly in dead cells. However, studies have shown that detection and quantification of mRNA are highly dependent both on expression levels of the target gene and on mRNA extraction protocols (Schmittgen 2001). Consequently, assays targeting different loci and/or involving different mRNA extraction protocols will perform differently (Sheridan et al. 1998; Norton and Batt 1999).

The main goal of this study was to develop a RT-PCR assay for *P. ramorum* and to test its sensitivity and specificity both in laboratory conditions and on a set of naturally infected samples. An assay was designed to detect the expression of the cytochrome oxidase gene subunit I (COX I), a large mitochondrial transmembrane protein that acts as the terminal enzyme of the respiratory chain: It catalyses the reduction of O_2 and simultaneously pumps protons across the membrane. Our hypothesis is that mRNA linked to respiration should be an ideal indicator of live target cells.

To examine the relationship between viability and the presence of mRNA, actively growing colonies of *P. ramorum* in Petri dishes were subjected to three different stress treatments, including heat exposure, rapid lyophilization and ethanol dipping. Treated samples were then tested by the new RT-PCR technique and by the well-established culturing and nested PCR techniques at varying time intervals after treatment. Finally, we compared the results from RT-PCR, culturing and two nested PCR assays on 100 symptomatic leaves of California bay laurel trees collected in five samplings in three different natural sites in California.

2 Material and methods

2.1 Development of the RT-PCR assay

2.1.1 Primer sequence design and specificity

Sequences of 18 different *Phytophthora* species were aligned using ClustalW (EMBL, European Bioinformatics Institute) to design the ACPramF/R primer set for the specific amplification of cDNA matching a 138-bp portion of the sequence of the mitochondrial cytochrome oxidase subunit I (COX 1) of *P. ramorum* (Fig. 1). Control 5.8S primer sequence was designed to amplify the 5.8S of both plants and oomycetes (Table 1). Primers were designed using Primer3 (http://www.embnet.sk/cgi-bin/primer3_www.cgi), and specificity of the primer set was preliminarily assessed by means of a Basic Local Alignment Search Tool (BLAST) search spanning across the entire NCBI data set.

2.1.2 RNA extraction

All samples used for RNA extraction were kept at −80°C before being extracted. RNA was extracted from approximately 50 mg mycelium from pure cultures (treated or untreated) or approximately 50 mg of symptomatic bay leaves using RNeasy® Plant Mini kit (Qiagen Inc., Valencia, CA, USA) for total RNA isolation from plants and filamentous fungi, following the manufacturer's protocol. Samples were first grounded in liquid nitrogen and then lysed under highly denaturating conditions. The RNase-free DNase Set (Qiagen) was used to purify RNA from DNA contamination.

2.1.3 Two-step RT-PCR

The presence of target COX I RNA was analysed by two-step RT-PCR using QuantiTect® Reverse Transcription kit (Qiagen) following the manufacturer's procedure. Each purified RNA sample was briefly incubated in gDNA Wipeout Buffer (Qiagen) at 42°C for 2 min to efficiently remove any possible contaminating genomic DNA. After genomic DNA elimination, reverse transcription was performed using RT Primer Mix (Qiagen), an optimized blend of oligo-dT and random primers that allows high cDNA yields from all regions of RNA transcript, even from 5′ regions. The entire reaction took place at 42°C for 15 min and was than inactivated at 95°C for 3 min. cDNA was then amplified using ACPramF/ACPramR primer set. PCR was performed in a total volume of 25 μl containing 6.26 μl of undiluted cDNA combined with 12.5 μl of 2× iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) containing 0.5 mM of each primer. Real-time amplification was carried out in an iCycler IQ Real-Time thermalcycler (Bio-Rad) using the following conditions: one cycle at 50°C for 10 min, one cycle at 95°C for 3 min, 35 cycles at 95°C for 15 s and 63°C for 1 min. Ramp rate was 3.3°C/s heating and 2.0°C/s cooling. PCR using 5.8S

Fig. 1. Multiple sequence alignment of selected primers utilized in COX I PCR of 18 different *Phytophthora* species. PCR primer sequences are shown in the first line. Colours underscore the homologous deletion breakpoint region whereas letters show the position of discrepancy between sequences from GenBank.

Table 1. Table of primer sets and fluorogenic probes used in this study.

Primer	Primer sequence (5′-3′)	Amplicon size	Location	Sense
ACPramF	CATTACCTGTTTTAGCTGGTGC	138	COXI	Forward
ACPramR	AAACTTCAGGGTGACCAAAA		COXI	Reverse
Phyto1	CATGGCGAGCGCTTGA	687	ITS1	Forward
Phyto4	GAAGCCGCCAACACAAG		ITS2	Reverse
Phyto2	AAAGCCAAGCCCTGCAC	291	ITS2	Forward
Phyto3	GGTGGATGGGGACGTG		ITS2	Reverse
Pram5	TTAGCTTCGGCTGAACAATG	73	ITS2	Forward
Pram6	CAGCTACGGTTCACCAGTCA		ITS2	Reverse
Pram7	FAM-ATGCTTTTTCTGCTGTGGCGGTAA-BHQ		ITS2	Probe
5.8SUnF	CTCGCACATCGATGAAGAAC	180	5.8S	Forward
5.8SUnR	GGAAGTGCAATATGCGTTCA		5.8S	Reverse

primers was performed using identical parameters. Immediately following amplification, product melt temperatures (T_m) were determined for each sample with the following conditions: 110 cycles at 62°C for 10 s, adding 0.3°C at each cycle (melt curve, data collection step). Ramp rates were 3.3°C/s heating and 2.0°C/s cooling. Both for the COX I and the 5.8S assays, only PCR amplification with threshold cycles (Ct) lower than 35 was considered positive. Sensitivity of the assay was tested on the following mRNA concentrations: 10 fg, 1, 100 pg, 1 and 10 ng.

2.2 Treatments to kill *P. ramorum*

Before processing the environmental samples, the stability of mRNA through time was determined by performing RT-PCR at different time intervals on putatively devitalized pure cultures of *P. ramorum*. At the same time, DNA of *P. ramorum* was also targeted (see below for extraction protocol) using both a nested SYBR Green (Hayden et al. 2004) and a nested Taqman (Hayden et al. 2006) assay. Isolates Pr1 (*Quercus agrifolia*, Marin County), Pr52 (*Rhododendron* sp., Santa Cruz County), Pr72 (*Rhododendron* sp., Santa Cruz County) and Pr102 (*Q. agrifolia*, Marin County) were employed for these tests. Each treatment thus included 12 plates.

Before treatment, mycelium of each isolate was grown in 20 ml of liquid pea broth (Cooke et al. 2000) at room temperature for 3 weeks. Treatments included (i) dipping mycelium in 70% EtOH for 1 h, (ii) exposure to 60°C for 1 h or (iii) placement of mycelium at −80°C for 2 h and overnight lyophilization. Treated mycelia and untreated controls were left at room temperature for 0, 1, 2, 5, 7, 9 and 12 days, and at the end of each time interval, treated and untreated mycelia from each flask were both plated onto four Petri dishes filled with V8 agar (Erwin and Ribeiro 1996) and placed at −80°C before proceeding with RNA extraction. Plates were checked 1 and 3 months after plating.

2.3 DNA extraction from pure cultures

Isolates of *P. ramorum* and other *Phytophthora* species tested (Table 2) were grown in 20 ml of pea broth (Cooke et al. 2000). After vacuum filtration, mycelia were freeze-dried for extended storage at −20°C. DNA was extracted from freeze-dried mycelium using a PureGene DNA Extraction kit (Gentra Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. DNA was suspended in high pressure layer chromatography water and stored at −20°C. For routine analysis, DNA was diluted to 10 μg/μl and maintained at 4°C.

2.4 Comparing diagnostic assays on field samples

2.4.1 Survey sites

The three sites selected for this study differ in their ecological and climatic parameters, but share as a common feature the presence of infected California bay laurel (*U. californica*). Samuel P. Taylor State Park (Long. −122.7361, Lat. 38.0296), a coast redwood forest mixed with tanoak (*Lithocarpus densiflorus*), is the most mesic site. Briones Regional Park (Long. −122.8295, Lat. 37.5641) is a xeric grassland and oak woodland made up of coast live oak (*Q. agrifolia*) and California black oak (*Quercus kelloggii*) mixed with bay laurel. This site is characterized by warm dry summers with limited incidence of fog. Finally, China Camp State Park (Long. −122.461, Lat. 38.0057) comprises woodlands prevalently composed of Pacific madrone (*Arbutus menziesi*), coast live oak, California black oak and bay laurel; it is an intermediate site between the moist (mesic) redwood forest and the dry (xeric) oak woodland.

2.4.2 Sampling and isolation

Samplings were carried out between October 2005 and July 2006. China Camp State Park was surveyed in October 2005, April and July 2006, whereas Samuel P. Taylor State Park and Briones Regional Park were surveyed only in July 2006. Each site was inspected for the presence of SOD, and symptomatic leaves from 20 California bay laurel trees, randomly chosen, were collected and taken to the laboratory.

A leaf from each tree was superficially wiped with 70% EtOH and then divided into three homogeneous parts containing approximately the same amount of lesions and healthy tissues. Each piece was used for isolation by direct plating on PARP selective medium (Erwin and Ribeiro 1996), DNA and RNA extraction, respectively, to compare different diagnostic methods. Asymptomatic California bay laurel leaves, collected from the U.C. Berkeley campus, were used as negative control.

2.4.3 DNA extraction from plant tissues and nested PCR

DNA was extracted from approximately 50 mg lyophilized bay leave tissue pulverized with two 6.35-mm glass beads (Biospec Products, Bartesville, OK, USA) in a FastPrep instrument (Bio 101, Carlsbed, CA, USA) for 5–30 s at 1800 oscillations per minute. Pulverized tissue was subjected to two repetitions of freezing (on dry ice for 2 min) and thawing (at 75°C for 2 min) in a 350 μl of CTAB buffer. DNA was extracted in phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and cleaned with the Geneclean® Turbo Nucleic Acid Purification kit (Qiagen Inc.) according to the manufacturer's protocol. DNA was eluted in 30 μl

Table 2. Phytophthora species used to determine specificity of reaction of the *Phytophthora ramorum* primers.

Species	Local isolate no.	Host	Origin	Amplification
P. cambivora[1]	MP14	*Quercus agrifolia*	California	Not cross-amplified
P. cambivora[1]	MP22	*Prunus*	California	Not cross-amplified
P. cambivora[1]	NY217	*Malus*	New York	Not cross-amplified
P. cambivora[1]	NY249	*Malus*	Oregon	Not cross-amplified
P. citricola[1]	MP18	*na*	California	Not cross-amplified
P. cryptogea[1]	MP11	*Lycopersicon esculentum*	*na*	Not cross-amplified
P. drechsleri[1]	*na*	*na*	*na*	Not cross-amplified
P. hibernalis[1]	1895	*Aquilegia vulgaris*	New Zealand	Not cross-amplified
P. nemorosa[2]	P16	*Umbellularia californica*	California	Amplified >1 pg
P. lateralis[2]	PL27	*Chamaecyparis lawsoniana*	California	Not cross-amplified
P. megasperma[1]	MP20	*Glycine max*	Wisconsin	Amplified >1 pg
P. palmivora[1]	MP8	*Theobroma cacao*	*na*	Amplified >1 pg
P. pseudosyringae[1]	P40	*Q. agrifolia*	California	Amplified >1 pg
P. syringae[1]	MP15	*Rhododendron spp.*	California	Not cross-amplified
P. ramorum[2]	Pr1	*Q. agrifolia*	California	Amplified >10 fg
P. ramorum[2]	Pr52	*Rhododendron sp.*	California	Amplified >10 fg
P. ramorum[2]	Pr72	*Rhododendron sp.*	California	Amplified >10 fg
P. ramorum[1]	Pr102	*Q. agrifolia*	California	Amplified >10 fg

na, data not available.
[1]Isolates used by Hayden et al. (2004).
[2]Isolates used by Hayden et al. (2006).

of Geneclean Elution Solution, and then, 3 μl of 1x TE buffer was added to preserve the extracts for long-term storage. DNA was diluted 1/100 before amplification. Nested PCR protocols for the SYBR green and Taqman assays were those described by Hayden et al. (2004, 2006).

2.5 Statistical analyses

All analyses were performed using the program JMP (SAS Institute Inc., Cary, NC, USA). Chi-square analyses were run to compare number of positive samples detected by different techniques both cumulatively and by individual sampling. Because not all symptomatic collected leaves were infected by *P. ramorum*, data were adjusted by excluding all samples negative by nested PCR, as it was assumed that these samples were not infected by the pathogen. When comparisons were made among three techniques, chi-square tests were performed using Pearson's tests. When *ad hoc* comparisons were made between results provided by two techniques, one-tailed Fisher's exact tests were employed instead.

3 Results

3.1 Specificity and sensitivity of RT-PCR primers

When sensitivity and specificity of the newly designed RT-PCR primers were tested on 18 isolates belonging to 12 *Phytophthora* species, the assay could detect 10 fg of mRNA from each of four *P. ramorum* isolates tested, while no amplification was obtained for seven other *Phytophthora* species at any of the tested DNA concentrations (Table 2). Although cross-reactivity was obtained at times with four other *Phytophthora* species, namely *P. nemorosa*, *P. palmivora*, *P. pseudosyringae* and *P. megasperma*, this occurred only when DNA was at concentrations higher than 1 pg/μl. Control 5.8S mRNA could be detected from plant tissue in all samples tested. PCR amplifications were performed using SYBR green, which allows for a real-time detection of amplified double-stranded DNA and for verification of the nature of the amplicon by determination of its melting temperature. Melt temperatures for 5.8S and COX I amplifications products were 78.6°C (SD = 0.29), and 82.2°C (SD = 0.23), respectively (Fig. 2).

3.2 Detection of mRNA from dead cells

Exposure of *P. ramorum* colonies to 60°C for 1 h only reduced the growth rate of the pathogen, but did not kill the mycelium, and all plates displayed active growth a week after treatment. Dipping the mycelium in ethanol 70% for 1 h caused a temporary complete stasis of the mycelial growth for 3 weeks after treatment; however, 50% of colonies of isolate Pr52 restarted growing actively 1 month after treatment. RT-PCR results were always positive for all heat-treated and ethanol-dipped cultures, confirming that such treatments had not effectively killed the pathogen. Rapid lyophilization, however, did effectively kill all mycelia of *P. ramorum*; thus, this treatment was selected for a comparative analysis of decay time of mRNA and DNA targeted by diagnostic assays. While nested PCR using the protocols of Hayden et al. (2004, 2006) could detect DNA 3 months after lyophilization, mRNA was only detected only up to 1 week after the treatment. Control 5.8S mRNA was detected in all samples, with the exclusion of water negative controls.

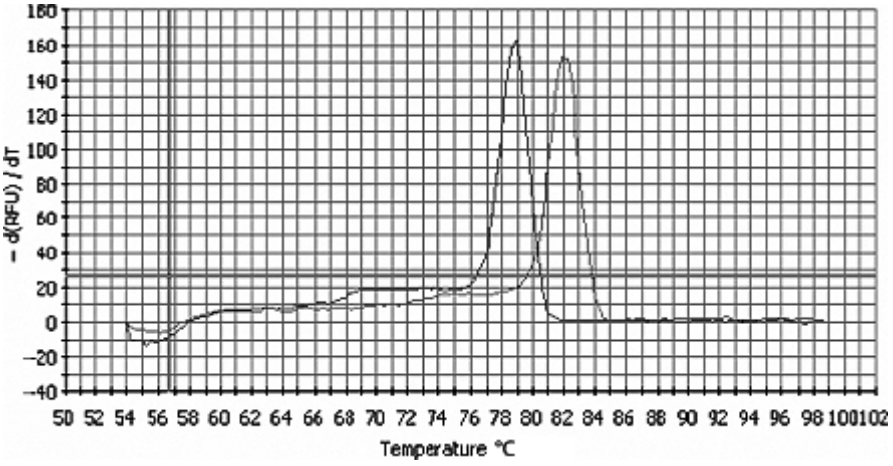

Fig. 2. Melting curve analysis was performed on RT-PCR products achieved using COXI primers and template from *Phytophthora ramorum* pure cultures and from naturally infected bay laurel leaves. Analysis confirmed the specificity of the PCR and the absence of primer dimers. The 78.6 and 82.2°C peaks represent positive mRNA detection of the 5.8S and COXI genes, respectively.

3.3 Comparison of methods for the study of viability in the field

Results from all samples combined indicated that there were significant differences among the four diagnostic methods ($p < 0.0001$; Table 3). The two nested PCR protocols were the most sensitive assays and were statistically indistinguishable from one another; hence, all comparisons were made with the nested SYBR green protocol of Hayden et al. (2004). Overall, the isolation approach was the least sensitive of the methods and detected *P. ramorum* only in 49% of leaves positive by nested PCR. The RT-PCR assay detected a number of positive *P. ramorum* samples that was intermediate between the two other approaches (69% of nested PCR positives) and differed significantly from both isolation ($p = 0.017$) and nested PCR ($p < 0.0001$) assays. Furthermore, all samples that were positive by isolation on PARP resulted positive with all three molecular methods, and all RT-PCR-positive samples were positive with DNA-based diagnostic methods.

While all assays tested were equivalent in the April sampling of China Camp, in all other samplings, performed during dry months, nested PCR detected *P. ramorum* in a significantly larger number of samples than the other assays (p range: 0.02–<0.0001). The number of *P. ramorum*-positive leaves by RT-PCR was significantly higher than that by culturing in the xeric Briones site sampled in July ($p = 0.0008$). Analyses from Samuel P. Taylor were not statistically significant because of the small number of leaves positively identified as infected by *P. ramorum*, but the trend was similar to that recorded at the other sites sampled in the dry season.

4 Discussion

This study demonstrates that mRNA is a promising indicator of viability of *P. ramorum*. At the tested conditions, mRNA was detected only for a week after cultures of *P. ramorum* were killed by rapid lyophilization, while DNA of the pathogen was detected even 3 months after the same treatment. Positive results obtained using the RT-PCR assay here described may thus indicate the presence of a pathogen isolate that is either alive or recently dead. The application of any molecular diagnostic approach directly on field samples requires a highly sensitive assay and justifies the choice of a multiple copy gene, such as COXI. Although cross-reactivity of the primers tested in this study was detected with a few other species, the main purpose of this study was to test whether mRNA detection may help confirm or deny viability of the pathogen in samples positively diagnosed as infected by *P. ramorum* through DNA-based tests. Additionally, cross-reactivity may only occur when RNA concentrations are relatively high, a condition not likely to occur in samples from nature. If absolute specificity is required, samples can always be tested using one of many DNA-based PCR assays to confirm that *P. ramorum* is present and other *Phytophthora* species absent.

It is generally accepted that DNA-based diagnostic techniques are extremely sensitive to the presence of target DNA, but do not differentiate between living and dead target cells. It has also been reported that culturing success of *P. ramorum* varies extremely depending on year, season, climatic conditions of the site and host substrate (Hayden et al. 2004; Vettraino et al. 2009). Thus, in unfavourable climatic conditions, culturing success is extremely low because of pathogen dormancy, and probably, it is not a good indicator of presence/absence of the pathogen.

In this study, all culture-positive samples were also RT-PCR positive, and all RT-PCR-positive samples were positive through nested PCR. Samples that were negative through nested PCR were negative by all other assays and were not included in the analysis, as they most likely represent leaves infected by foliar pathogens other than *P. ramorum*. The nested configuration of the data allows to draw some inferences on pathogen viability based on the differential results provided by each diagnostic assay. Cumulatively, diagnostic success of RT-PCR was always intermediate between culturing and nested PCR. RT-PCR was shown to be statistically different from either culturing or nested PCR. Our data are suggestive that 31% (n = 24) samples positive by nested PCR but negative by RT-PCR may represent isolates that either have recently died or produce mRNA under the threshold of detection of the assay. On the other hand, 20% (n = 15) samples that are culture negative but RT-PCR positive are likely to be dormant, but still alive, in spite of their lack of growth *in vitro*.

Table 3. Percentage of symptomatic bay laurel leaves from which *Phytophthora ramorum* was identified by direct plating on PARP selective medium, RT-PCR, Nested TaqMan PCR and Nested SYBR Green PCR. Each sampling consisted of 20 leaves, but only SYBR Green positive samples (in parentheses) were used in the calculations. Pearson's chi-square was utilized to compare frequencies of positive *P. ramorum* detection among nested SYBR Green, RT-PCR and PARP plating. One-tailed Fisher's exact tests were employed to compare nested SYBR Green to RT-PCR, and RT-PCR to PARP plating.

	China camp			Briones	Samuel P. Taylor	
Methods	October	April	July	July	July	All samples
PARP	41	100	40	7	50	49
RT-PCR	53	100	45	67	83	69
TaqMan	94	100	100	100	100	99
SYBR Green	100 (17)	100 (20)	100 (20)	100 (15)	100 (6)	100 (78)
p of Pearson's chi-square	<0.0007	n.s.	<0.0001	<0.0001	n.s.	<0.0001
Fisher's exact tests						
p SYBR>RT-PCR	0.001		<0.0001	0.02		<0.0001
p RT-PCR>PARP	n.s.		n.s.	0.0008		0.017

This study also highlights that the relative diagnostic power of each of the three assays may differ based on time of sampling and study site. The Spring of 2006 was rainy and conducive to active infestations by *P. ramorum* (Rizzo et al. 2005); hence, it is no surprise that samples collected in April were 100% positive, independent of assay employed. However, in the dry months of October 2005 and July 2006, the three approaches were not equivalent, and nested PCR assays identified *P. ramorum* in a number of samples significantly larger than RT-PCR. During the dry season, thus, nested PCR may definitely overestimate the presence of the pathogen by detecting its DNA even when the isolate is no longer viable. Finally, the advantage of RT-PCR over culturing for the detection of viable infections was strongly demonstrated by the results from Briones Regional Park. In this particularly xeric site, culturing success was extremely low (7%), but RT-PCR success was significantly ($p = 0.0008$) higher (67%). Overall, these results suggest that culturing and DNA-based diagnosis of *P. ramorum* may be acceptable in mesic sites and in years climatically favourable to the pathogen, but in dry sites and/or in years climatically unfavourable to the pathogen, culturing may greatly underestimate the number of viable samples, while DNA-based detection may overestimate that number. RT-PCR appears to be a better indicator of viability of *P. ramorum*, providing consistent results that are independent of climatic conditions or seasonal patterns. Because *P. ramorum* is seasonally active and RT-PCR was less variable than culturing in the detection of viable infections, we conclude that mRNA has the potential to be the most appropriate target for assays to be employed not only for diagnosis and detection but also for studies on the ecology of *P. ramorum* and of other *Phytophthora* species as well as on the epidemiology of polycyclic diseases. Finally, although mRNA quantification was not attempted in this study, it may be tested in the future using this technique. Quantitative RT-PCR may be an approach to better differentiate between active, dormant and recently dead cells.

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