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On-Site DNA Extraction and Real-Time PCR for Detection of *Phytophthora ramorum* in the Field

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Phytophthora ramorum **is a recently described pathogen causing oak mortality (sudden oak death) in forests in coastal areas of California and southern Oregon and dieback and leaf blight in a range of tree, shrub, and herbaceous species in the United States and Europe. Due to the threat posed by this organism, stringent quarantine regulations are in place, which restrict the movement of a number of hosts. Fast and accurate diagnostic tests are required in order to characterize the distribution of *P. ramorum*, prevent its introduction into pathogen-free areas, and minimize its spread within affected areas. However, sending samples to a laboratory for testing can cause a substantial delay between sampling and diagnosis. A rapid and simple DNA extraction method was developed for use at the point of sampling and used to extract DNAs from symptomatic foliage and stems in the field. A sensitive and specific single-round real-time PCR (TaqMan) assay for *P. ramorum* was performed using a portable real-time PCR platform (Cepheid SmartCycler II), and a cost-effective method for stabilizing PCR reagents was developed to allow their storage and transportation at room temperature. To our knowledge, this is the first description of a method for DNA extraction and molecular testing for a plant pathogen carried out entirely in the field, independent of any laboratory facilities.**

Phytophthora ramorum is the causal agent of extensive oak mortality (commonly known as sudden oak death) in coastal forests in California (27) and southern Oregon (12, 25). This pathogen also causes ramorum leaf blight and dieback on a range of other plant species (9) and can have a profound and devastating effect on forest ecosystems. A distinct population of the same pathogen (6, 35) is found in a number of European countries (10, 20, 23, 37), mostly causing dieback and leaf blight on a range of ornamental plants in nurseries and landscaped areas (2). There have also recently been a number of incidences of lethal bark cankers caused by *P. ramorum* in native and nonnative trees in Europe (7). *P. ramorum* has a broad and expanding host range (3, 4, 10, 11, 19, 20, 28, 31), and as a result of the threat posed to forest ecosystems, the movement of a variety of its host species is subject to restrictions in Europe and the United States. Emergency European Community phytosanitary measures for *P. ramorum* were introduced in 2002 (1), and in the United States quarantine restrictions at both the federal and state levels control the movement of a variety of plant species from infested areas in California and Oregon (31). The availability of rapid and accurate detection methods for *P. ramorum* is critical to allow its prevalence to be monitored and to expedite management or eradication steps to prevent its introduction and minimize its spread.

The identification of *P. ramorum* is not possible based on host symptoms alone due to the considerable variation in their expression and because a range of other causes can produce similar symptoms. These include infections by several other *Phytophthora* spp. which are also commonly recovered from symptomatic plant material, including leaf and twig lesions and trunk cankers (22). *P. ramorum* has a characteristic morphology which allows it to be distinguished from other *Phytophthora* spp. when isolated in culture; however, culturing of the pathogen from symptomatic plant material is time-consuming, and under some circumstances its success may be dependent on the species of the host or the environmental conditions from which the sample was taken (22). Furthermore, despite *P. ramorum*'s distinctive morphological characteristics, the identification of an unknown culture solely on the basis of morphology requires specialist training and experience. PCR-based techniques have been developed for the diagnosis of a wide range of plant pathogens, and a number of molecular detection methods have been developed which can distinguish between *P. ramorum* and other *Phytophthora* spp. with high levels of specificity and sensitivity (14, 18, 22). Real-time PCR methods can have advantages of speed, accuracy, and sensitivity over conventional PCR-based techniques (29) and can be based on a range of different detection chemistries. A nested real-time PCR assay for *P. ramorum* using SYBR green (a double-stranded DNA-binding dye [as DNA is amplified, more dye is bound and thus fluoresces]) has been described (14), and an assay using molecular beacons (probes containing reporter and quencher dyes which hybridize to the amplified product, resulting in increased fluorescence) is in development (5). Real-time PCR methods based on TaqMan chemistry (amplification-dependent cleavage of probes incorporating reporter and quencher dyes, resulting in increased fluorescence) have the particular advantage of requiring no postamplification steps and therefore involve a reduced risk of cross-contamination, and they have been described for a wide range of plant pathogens (26, 30, 32, 33, 34). A single-round TaqMan PCR assay for the detection of *P. ramorum* has recently been developed which compares extremely favorably with morphological methods of identification (K. J. D. Hughes, R. L. Griffin, J. A. Tomlinson, N.

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Boonham, A. J. Inman, and C. R. Lane, submitted for publication). This assay is routinely used at the Central Science Laboratory (CSL) in the United Kingdom, in conjunction with isolation techniques, for the detection of *P. ramorum* in symptomatic plant material in the laboratory.

Sending samples to a central laboratory facility for testing has disadvantages, in particular the length of time between sampling and diagnosis, and in some circumstances it would be highly desirable to perform testing immediately at the point of sampling. In particular, on-site testing would permit the targeted testing of known *P. ramorum* hosts, such as imported nursery stock, at points of entry with minimal disruption to trade. Symptoms caused by *P. ramorum* infection are often not diagnostic, and it has been estimated (C. R. Lane, personal communication) that typically only 20% of suspect samples submitted for laboratory testing will be shown to be infected with *P. ramorum* or other *Phytophthora* species of quarantine concern. On-site testing partially obviates the need to hold suspect material (the majority of which will test negative) while waiting for a laboratory test result. This has important implications in maintaining the credibility of a plant health exclusion policy designed to protect native flora while minimizing any undue disruption to legitimate trade in plant material. Any samples which test positive in the field can still be sent to a diagnostic laboratory for confirmation or further characterization. Portable real-time PCR platforms have been developed, including the SmartCycler instrument (Cepheid, Sunnyvale, CA), the R.A.P.I.D. system and RAZOR instrument (Idaho Technologies, Salt Lake City, UT), and the BioSeeq instrument (Smiths Detection, Edgewood, MD), which are designed for on-site molecular testing. Use of the SmartCycler has been described for on-site detection of the bacterium *Xylella fastidiosa* in grape plants (30) directly from sap and macerated chips of xylem. The molecular detection of fungal pathogens in plant material, however, requires the extraction of DNA (29), so on-site molecular testing demands not only a portable real-time PCR platform and suitable assay but also a simple and robust DNA extraction method which can be performed in the field.

This paper describes a method for DNA extraction from symptomatic plant material in the field and the use of a portable real-time PCR platform (Cepheid SmartCycler) for accurate on-site detection of *P. ramorum* within 2 h. The DNA extraction method can be completed within 30 min, and unlike many laboratory extraction methods, does not require centrifugation steps, organic solvents, or the use of liquid nitrogen for sample homogenization. PCR reagents were chosen to give the sensitivity and specificity necessary for testing infected plant material. A procedure for lyophilizing real-time PCR reagents was developed to allow their storage and transportation at room temperature and to simplify their use in the field. DNA extracts were tested using single-round multiplex real-time PCR (TaqMan), which is completed in just over 1 h. The use of a closed-tube single-round PCR assay greatly reduces the risk of false-positive results due to cross-contamination compared to that with nested or seminested PCR. In addition to *P. ramorum*-specific primers and probe, generic plant cytochrome oxidase (COX)-specific primers and probe were used to detect host DNA, providing confirmation that DNA extraction was successful and thereby avoiding false-negative results for *P. ramorum*. The protocol has been used outside the laboratory to extract and test DNAs from healthy and infected plants at disease outbreak sites several hundred miles from the diagnostic laboratory and can be performed in the field using equipment powered by a generator. The combination of an extraction method, real-time PCR assay, and reagents, all optimized for use in the field, allows the detection of *P. ramorum* in naturally infected material at the point of sampling, with comparable results to those of real-time PCR testing in the laboratory.

MATERIALS AND METHODS

***P. ramorum* inoculation of plant material.** *P. ramorum* isolates were grown on carrot piece agar (35) for 1 week, and then 0.5-cm^2 agar plugs were taken from the leading edge of colonies and used to inoculate wounded detached leaves of *Rhododendron ponticum*, which were then incubated at room temperature in a damp chamber for 1 week. Inoculations using other *Phytophthora* species were set up in the same way and incubated for 7 to 10 days.

DNA extraction from cultures. DNAs were extracted from cultures of *P. ramorum* and other *Phytophthora* species grown on P_5ARP-(H) (16) or carrot piece agar, using a NucleoSpin plant kit (Machery-Nagel, Düren, Germany) according to the manufacturer's protocol for fungi.

DNA extraction from plant material. DNA extraction was performed using a QuickPick Plant DNA kit and PickPen 8-M from Bio-Nobile (Turku, Finland), following the manufacturer's instructions for processing up to 24 samples in parallel in a 96-well microplate. Briefly, approximately 15 to 25 mg of tissue (for infected material, this was taken from the leading edge of a lesion) was placed into a plastic bag, frozen in liquid nitrogen, ground to a powder using a small hand roller, and transferred to a microcentrifuge tube containing 35 μl of plant DNA lysis buffer. Plant DNA proteinase K solution (5 μl) was added, and the sample suspension was vortexed and then incubated at 65°C for 20 to 30 min in a heating block. After incubation, the sample was centrifuged at maximum speed (approximately 18,000 × *g*) in a benchtop microcentrifuge for 5 min. The supernatant was transferred to a well in the first row of a 96-well standard microplate (ABgene, Epsom, United Kingdom) containing plant DNA MagaZorb magnetic particles (5 μl) and plant DNA binding buffer (60 μl) and mixed by gentle shaking for 2 min. The PickPen was used to transfer the magnetic particles and bound DNA through two washing steps (150 μl plant DNA wash buffer) and into 100 μl plant DNA elution buffer, followed by incubation at room temperature for 5 min with occasional gentle mixing, using the PickPen with magnets withdrawn, and then removal of the magnetic particles.

Adaptations were made to the method in order to improve its suitability for use in the field. The length of the 65°C incubation was reduced, and extractions were performed with the centrifugation step omitted. Samples were also homogenized by grinding with a roller without prefreezing or were cut into pieces of <2 mm^2 using a scalpel blade. After the addition of proteinase K, samples were mixed by pipetting up and down or gently flicking the tube to remove the need for a vortexer. DNA extracts were tested using *P. ramorum*-specific and/or plant internal control primers and probes (described below) on an ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA).

Real-time PCR primers and probes. The *P. ramorum*-specific TaqMan primers (*Pram*-114F and *Pram*-190R) and probe (*Pram* probe) and generic 5.8S TaqMan primers (5.8S F and 5.8S R) and probe (5.8S probe) were designed based on internal transcribed spacer sequences (K. J. D. Hughes, R. L. Griffin, J. A. Tomlinson, N. Boonham, A. J. Inman, and C. R. Lane, submitted for publication). The plant internal positive control primers (COX F and COX R_W) and probe (COX probe) were based on a previously described assay designed for the cytochrome oxidase (COX) gene (34). Primer and probe sequences and reporter/quencher dyes are shown in Table 1. All primers and probes were synthesized by MWG Biotech (Ebersberg, Germany).

Real-time PCR. Real-time PCR was carried out in an ABI Prism 7700 or SmartCycler II instrument (Cepheid, Sunnyvale, CA). In all cases, 1 μl of DNA extract was added to 24 μl of master mix, and negative controls containing nuclease-free water instead of DNA were included in each run. DNA extracts were tested in duplicate, except where otherwise stated for individual experiments. The final primer concentrations were 300 nM for each *P. ramorum* primer and/or 200 nM for each COX primer, and probes were used at a final concentration of 100 nM. Real-time PCR was carried out using TaqMan core reagents (Applied Biosystems) consisting of 1× buffer A (50 mM KCl, 10 mM Tris-HCl, pH 8.3, carboxy-X-rhodamine [ROX] passive reference dye) and 0.025 U/μl

TABLE 1. Characteristics of primers and TaqMan probes

Primer or probe	Sequence (5′-3′)	Reporter (5′)[a]	Quencher (3′)[a]	Final conc (nM)
Pram-114F	TCATGGCGAGCGCTTGA			300
Pram-190R	AGTATATTCAGTATTTAGGAATGGGTTTAAAAAGT			300
Pram probe	TTCGGGTCTGAGCTAGTAG	FAM	TAMRA or BHQ1	100
COX F	CGTCGCATTCCAGATTATCCA			200
COX R_W	CAACTACGGATATATAAGRRCCRRAACTG			200
COX probe	AGGGCATTCCATCCAGCGTAAGCA	JOE or TET	TAMRA or BHQ1	100
5.8S F	TGTCTAGGCTCGCACATCGA			300
5.8S R	GATGACTCACTGAATCCTGCAATT			300
5.8S probe	ACGCTGCGAACTGCGATACGTAATGC	JOE	BHQ1	100
Pram-114Fc	TCATGGCGAGCGCTGGA			300

[a] FAM, 6-carboxyfluorescein; TAMRA, tetramethylcarboxyrhodamine; BHQ1, black hole quencher 1 (Biosearch Technologies, Novato, CA); JOE, 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein; TET, tetrachloro-6-carboxyfluorescein.

Ampli*Taq* Gold plus 0.2 mM (each) deoxynucleoside triphosphate and 5.5 mM $MgCl_2$. The cycling conditions were 10 min at 95°C followed by 40 two-step cycles of 15 s at 95°C and 1 min at 60°C. Trehalose (Sigma, St. Louis, MO) was added to some reactions to give a final concentration of 5% (wt/vol).

Results were analyzed in terms of cycle threshold (C_T) values. As amplification occurs in a TaqMan reaction, the 5′ exonuclease activity of *Taq* polymerase (15) results in cleavage of the dual-labeled probe (21) and hence an increase in reporter fluorescence, which is monitored in real time. The C_T value is the cycle at which the fluorescence signal exceeds a threshold value, so a reduction in C_T represents an improvement in performance. A C_T value of <40 cycles was regarded as a positive result, and a negative result is represented by a C_T value equal to 40. Default threshold settings were used on the ABI Prism 7700 (10 standard deviations above the mean fluorescence generated during cycles 3 to 15) and SmartCycler (30 fluorescence units) instruments.

Stabilization of reagents. Reagents were freeze-dried in 5-ml glass freeze-drying vials with rubber stoppers (Fisher Scientific, Hampton, NH). A master mix containing all reagents (except template DNA) was divided into aliquots in vials, frozen at −20°C, and freeze-dried in a Modulyo freeze dryer (Thermo, Milford, MA) for at least 6 h or overnight before being sealed under a vacuum and stored at room temperature in the dark. Each vial contained sufficient reagents for 10 real-time PCRs, and the contents were resuspended in 240 μl of nuclease-free water (Promega, Madison, WI) before use.

Testing in the field. When the assay was performed in the field, steps were taken to avoid contamination and to increase convenience and speed. Plant samples were placed in small disposable plastic weigh boats, cut into 1- to 2-mm^2 pieces using a scalpel blade, and then transferred into tubes containing lysis buffer. A new weigh boat and scalpel blade were used for each sample, and gloves were worn throughout and changed between samples to prevent cross-contamination. All components of the QuickPick Plant DNA kit except proteinase K were previously divided into aliquots in the laboratory. Plant DNA lysis buffer was divided into aliquots in 0.6-ml microcentrifuge tubes, and the remaining buffers (binding buffer plus MagaZorb magnetic particles, wash buffer, and elution buffer) were divided into aliquots in 96-well microplates and heat sealed with Easy Peel heat-sealing foil (ABgene). To ensure nuclease-free conditions and to avoid contamination, sterile filter pipette tips were used throughout. DNA extraction and reaction setup were performed in separate locations depending on the nature of the testing site. For example, when the testing was performed in a vehicle, extraction was performed in the front seat and PCRs were set up in the back. Under such circumstances, the heating block and SmartCycler were powered by a generator in the absence of mains electricity.

RESULTS

DNA extraction. The effect of modifications to the extraction method was examined by comparing C_T values for DNAs extracted with and without each modification. DNAs were extracted from identical samples of *P. ramorum*-infected *R. ponticum* leaves and tested using *P. ramorum*- and COX-specific primers and probes in multiplex reactions. For each modification, two extracts were prepared using the modified extraction method and one extract was simultaneously prepared using the unmodified protocol. All extracts were tested in duplicate on the ABI Prism 7700, and mean C_Ts were calculated for the modified protocol (two extracts, each tested in duplicate [$n = 4$]) and the unmodified protocol ($n = 2$). The C_T difference was calculated by subtracting the mean C_T value without modification from the mean C_T value with modification, and the estimated standard error for the C_T difference was calculated. Omission of the centrifugation step resulted in a small increase, of 1.04 ± 0.42, in the *P. ramorum* C_T value, and reducing the incubation time to 10 min resulted in a small decrease, of 0.87 ± 0.13, in the *P. ramorum* C_T value. DNAs were also extracted using a range of grinding methods. No detectable *P. ramorum* DNA was extracted from samples which were ground without being prefrozen in liquid nitrogen (*P. ramorum* C_T value = 40). Amplifiable *P. ramorum* DNA was successfully extracted, however, from samples which had been cut into pieces using a scalpel blade instead of being ground (*P. ramorum* C_T increased by 2.94 ± 0.81 compared to that for samples ground with prefreezing). These results show that the centrifugation step is not critical and can reasonably be excluded from the protocol to remove the need for a microcentrifuge in the field and that the incubation time can be reduced to 10 min with no adverse effects. Also, it is possible to successfully extract amplifiable *P. ramorum* DNA from samples without using liquid nitrogen, by cutting material instead of grinding it. When the centrifugation step is omitted, cutting samples instead of grinding them also allows the supernatant to be more easily transferred by pipetting following proteinase K digestion. The modifications to the extraction protocol had similar effects on COX C_T values (Fig. 1), and similar results were also observed for healthy *R. ponticum* leaves and healthy and *P. ramorum*-infected *R. ponticum* stems (data not shown). It was concluded that despite the small decrease in extraction efficiency observed when plant material was cut instead of ground, this method of sample preparation would be suitable for use in the field, as it has the significant advantage of allowing small samples to be processed rapidly and without the need for liquid nitrogen.

Probe selection for use with the SmartCycler. The *P. ramorum* real-time PCR assay performed in the laboratory on an ABI Prism 7700 uses a *P. ramorum*-specific probe with a 6-carboxyfluorescein reporter and a 6-carboxyltetramethylrhodamine (TAMRA) quencher and a COX-specific probe with a

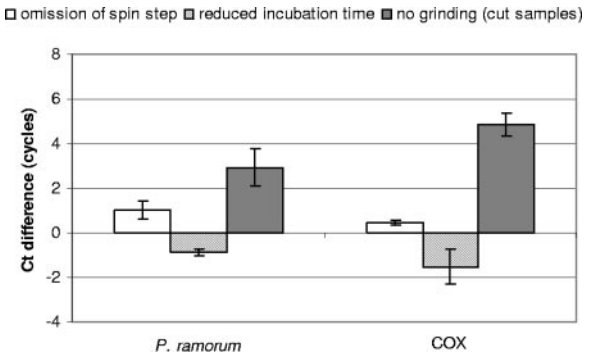

FIG. 1. Effects of modifications to the PickPen DNA extraction protocol on C_T values. DNAs were extracted from samples of *P. ramorum*-inoculated *R. ponticum* and tested with *P. ramorum* and COX primers and probes. For each modification, the results shown are for duplicate extracts using the modified method and single extracts using the unmodified method, with each tested in duplicate. C_T differences were calculated by subtracting the mean C_T value for the method without modification from the mean C_T value for the method with modification. Negative C_T differences represent improved performances. Error bars show estimated standard errors for C_T differences.

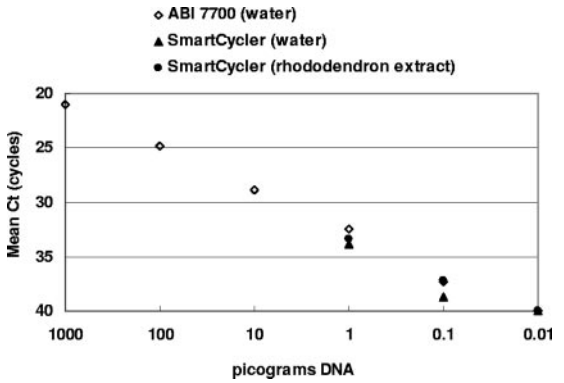

FIG. 2. Limit of detection of *P. ramorum* primers (*Pram*-114F and -190R) and probe. A solution of *P. ramorum* DNA containing approximately 1 ng DNA per μl was serially diluted in water or in a solution of DNA extracted from a *P. ramorum*-negative rhododendron. Dilutions were tested in duplicate on an ABI Prism 7700 and/or SmartCycler II.

6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein (JOE) reporter and a TAMRA quencher (Table 1). However, the optical system of the SmartCycler necessitates the use of differently labeled probes. The quencher dye TAMRA emits a signal (emission maximum, 568 nm) which is read in channel 2 of the SmartCycler's four optical channels, so TAMRA was replaced with a nonfluorescent quencher in order to allow a reporter dye to be accurately measured in this channel. DNA extracted from a *P. ramorum*-infected rhododendron leaf was tested in duplicate on the ABI Prism 7700 and the SmartCycler, using the *P. ramorum*-specific primers 114F and 190R and the *P. ramorum* probe with either a fluorescent quencher (TAMRA) or a nonfluorescent quencher (BHQ1). The C_T values observed for both probes with both machines were all within a range of 0.7 cycle (24.56 to 25.26). In addition, the SmartCycler is calibrated to detect tetrachloro-6-carboxyfluorescein (TET) (emission maximum, 535 nm) in channel 2 rather than JOE (emission maximum, 548 nm), and thus a COX probe with a TET reporter and a BHQ1 quencher was used with COX primers to test the same DNA extract (also in duplicate). The TET-BHQ1 probe gave slightly lower C_T values with the SmartCycler (31.37; standard deviation, 0.07) than those observed with the ABI Prism 7700 and a JOE-TAMRA probe (32.31; standard deviation, 0.16).

Limit of detection. DNA extracted from a culture of *P. ramorum* was quantified spectrophotometrically, and a dilution series was tested in duplicate using *P. ramorum* primers 190R and 114F with the ABI Prism 7700 and SmartCycler machines. With both machines, the assay was able to detect approximately 100 fg total DNA. DNA extracted from *P. ramorum* was also diluted in healthy rhododendron leaf extract (prepared using the on-site extraction protocol) and tested on the SmartCycler. No decrease in sensitivity was observed (Fig. 2), suggesting that although rhododendrons are high in polyphenolics, which can cause an inhibition of PCR, rhododendron extracts prepared using the on-site method did not contain sufficient inhibitors to significantly affect the PCR. DNAs extracted from artificially and naturally infected rhododendron leaves using the on-site extraction method were typically observed to give C_T values of approximately 25 to 30 (e.g., see Fig. 5), so it can be inferred that 1 μl of DNA extracted from 15 to 25 mg of symptomatic rhododendron leaf using the on-site extraction method contains in the region of 10 to 100 pg of *P. ramorum* DNA, which is approximately 100- to 1,000-fold higher than the threshold of detection.

Specificity. The on-site *P. ramorum* assay was used to test a total of 59 isolates of 30 species of *Phytophthora* (Table 2). Thirty isolates of 21 species, including several which are known or likely to infect rhododendrons or which can produce similar symptoms to those caused by *P. ramorum*, were inoculated onto wounded leaves of *Rhododendron ponticum*. Necrotic lesions were produced by isolates of seven species which are known to affect rhododendrons (*Phytophthora cactorum*, *Phytophthora citricola*, *Phytophthora cryptogea*, *Phytophthora heveae*, *Phytophthora kernoviae*, *P. ramorum*, and *Phytophthora syringae*) and also by two species which are not known to affect rhododendrons (*Phytophthora botryosa* and *Phytophthora macrochlamydospora*). DNAs were extracted from lesions, using the on-site extraction method, and were tested using the *P. ramorum*- and COX-specific primers and probes in multiplex reactions on the SmartCycler. All extracts tested positive for COX (C_T values, 22.29 to 33.95), demonstrating that the DNA extractions had been successful, and only DNAs extracted from *P. ramorum*-inoculated leaves gave *P. ramorum* C_T values of <40. DNAs were also extracted from cultures of all 59 isolates and tested with generic 5.8S primers and probe and *P. ramorum* primers and probe on the ABI Prism 7700. All extracts gave 5.8S C_T values between 13.44 and 18.69 cycles (corresponding to at least 100 ng of DNA per 25-μl reaction for each extract). *Phytophthora lateralis* was the only species other than *P. ramorum* which gave a *P. ramorum* C_T value of <40 (Table 2). However, no cross reaction with *P. lateralis* was observed when the extracts were diluted to give a concentration comparable to that in an on-site plant extract (approximately 100 pg/μl), and the limit of detection for *P. lateralis* was 1 to 10 ng

TABLE 2. Reactivity of *Phytophthora* isolates with generic 5.8S- and *P. ramorum*-specific primers and probes
Country of origin is shown for *P. ramorum* isolates.

Species	Isolate reference	5.8S		*P. ramorum*	
		Mean C_T value	Result	Mean C_T value	Result
P. boehmeriae	P 6950[a]	16.28	+	40.00	−
	CBS 100410[b]	16.56	+	40.00	−
P. botryosa	P 6945[a]	18.04	+	40.00	−
P. cactorum	MUCL9638[c]	17.93	+	40.00	−
	CSL 2151[d]	18.09	+	40.00	−
P. cambivora	CBS 376.61[b]	16.60	+	40.00	−
	FR P315[e]	17.06	+	40.00	−
P. cinnamomi	SCRI CIN5[f]	16.03	+	40.00	−
	PD 93/1389[g]	16.23	+	40.00	−
P. citricola	CSL 1531[d]	16.25	+	40.00	−
	CSL 2098[d]	16.43	+	40.00	−
	SCRI CIT1[f]	16.86	+	40.00	−
	CSL 1530[d]	17.07	+	40.00	−
	CBS379.61[b]	17.14	+	40.00	−
P. citrophthora	IMI 132217[h]	16.07	+	40.00	−
P. cryptogea	SCRI P521[f]	16.95	+	40.00	−
P. erythroseptica	P 7889[a]	18.02	+	40.00	−
P. europaea	CBS 109053[b]	17.39	+	40.00	−
P. fragariae var. rubi	FR-163[i]	16.77	+	40.00	−
P. gonopodyides	P 10337[a]	16.28	+	40.00	−
P. heveae	CBS 958.87[b]	17.04	+	40.00	−
P. hibernalis	P 3822[a]	17.45	+	40.00	−
P. ilicis	P 3939[a]	17.37	+	40.00	−
P. insolita	P 6195[a]	17.65	+	40.00	−
P. kernoviae	FR CAE36B[e]	16.94	+	40.00	−
	CSL 2169[d]	16.98	+	40.00	−
	CSL 2306[d]	17.15	+	40.00	−
	FR CAE4[e]	17.61	+	40.00	−
	FR P1560[e]	17.88	+	40.00	−
P. lateralis	P 1728[a]	16.96	+	31.07	+
	P 3888[a]	18.69	+	32.55	+
P. macrochlamydospora	P 10263[a]	17.10	+	40.00	−
P. megasperma	CBS 320.49[b]	16.26	+	40.00	−
P. nemorosa	P 10288[a]	17.18	+	40.00	−
P. nicotianae	CBS 411.87[b]	17.72	+	40.00	−
P. palmivora	SCRI P488[f]	17.50	+	40.00	−
P. nicotianae	SCRI NIC1[f]	16.48	+	40.00	−
P. pseudosyringae	CSL2369[d]	15.79	+	40.00	−
	P 10444[a]	16.15	+	40.00	−
P. quercina	P 10334[a]	17.10	+	40.00	−
P. ramorum (Germany)	BBA 69082[j]	14.74	+	11.55	+
	BBA 14/98a[j]	15.57	+	12.10	+
	BBA 13/99-1[j]	14.09	+	10.64	+
	BBA 15/01/46[j]	14.18	+	10.96	+
P. ramorum (UK)	CSL 1677[d]	13.49	+	10.37	+
	CSL 1678[d]	14.12	+	10.21	+
	CSL 1671[d]	13.44	+	10.41	+
P. ramorum (USA)	P1403[k]	17.01	+	13.87	+
	P1404[k]	14.85	+	11.55	+
	P1348[e]	14.07	+	10.30	+
	P1430sz[e]	14.53	+	11.16	+
P. richardiae	P 7788[a]	17.12	+	40.00	−
	P 10335[a]	17.77	+	40.00	−
	P 7789[a]	17.80	+	40.00	−
P. syringae	CBS 272.55[b]	16.34	+	40.00	−
	4N0247-5[i]	16.53	+	40.00	−
	4N0247-6[j]	16.93	+	40.00	−
	CBS 364.52[b]	17.84	+	40.00	−
P. uliginosa	CBS 109055[b]	17.25	+	40.00	−

[a] From Michael Coffey, University of California.
[b] From Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.
[c] From Belgian Coordinated Collections of Microorganisms.
[d] From Central Science Laboratory, York, United Kingdom.
[e] From Alice Holt, Forest Research, Farnham, United Kingdom.
[f] From David Cooke, Scottish Crop Research Institute (SCRI), Invergowrie, Dundee, United Kingdom.
[g] From Hans de Gruyter, Dutch Plant Protection Service, Wageningen, The Netherlands.
[h] From CABI Bioscience, Egham, United Kingdom.
[i] From Laboratoire National de la Protection des Vegataux, Nancy, France.
[j] From Sabine Werres, Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Braunschweig, Germany.
[k] From Everett Hansen, University of Oregon.

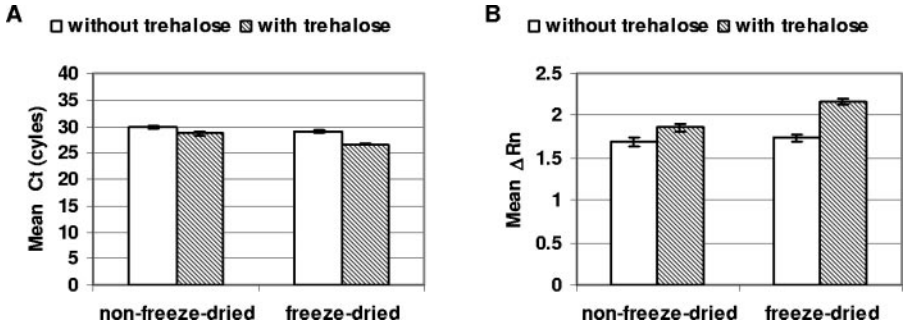

FIG. 3. Effect of trehalose on C_T values and fluorescence (ΔR_n) for frozen and freeze-dried master mixes. A complete PCR master mix containing *P. ramorum* primers and probe, with or without the addition of 5% trehalose, was freeze-dried and used to test DNA extracted from *P. ramorum*-inoculated *R. ponticum*. (A) C_T values; (B) ΔR_n values. The data shown are mean values for six replicate reactions; error bars show standard deviations.

DNA/25-μl reaction (data not shown), compared with 10 to 100 fg for *P. ramorum*.

Stabilization of real-time PCR reagents. A master mix containing buffer A, $MgCl_2$, deoxynucleoside triphosphates, Ampli*Taq* Gold, *P. ramorum* primers, and the *P. ramorum* probe was freeze-dried in vials, with and without the addition of trehalose at a final concentration of 5%. The contents of each vial were rehydrated in nuclease-free water before use. DNA extracted from a *P. ramorum*-infected rhododendron leaf was tested on an ABI Prism 7700 instrument (six replicate reactions for each master mix), using the freeze-dried master mix and non-freeze-dried reagents, with and without trehalose, and the C_T values were compared. The addition of trehalose reduced the C_T values for both freeze-dried and non-freeze-dried reagents and increased the end-point fluorescence (ΔR_n). The lowest C_T value and the highest ΔR_n measured were for master mix that had been freeze-dried in the presence of trehalose (Fig. 3).

Long-term storage of stabilized real-time PCR reagents. Batches of complete master mix (containing 5% trehalose) were frozen at −20°C or freeze-dried in vials and stored at room temperature for 5 months. Master mixes were tested on the ABI Prism 7700, using aliquots of the same DNA extract from a *P. ramorum*-inoculated rhododendron (six replicate reactions), which were stored at −20°C before use. Very similar C_T values were observed for freeze-dried and frozen master mixes for up to 5 months after the mixes were made, and ΔR_n values were slightly higher for the freeze-dried master mix (Fig. 4). The freeze-dried master mix was tested after a further month, and no increase in the C_T value or decrease in ΔR_n was observed. An analysis of raw fluorescence data for individual dye components revealed that the increased ΔR_n values recorded for freeze-dried master mix could be attributed to decreased passive reference (ROX) signals after freeze-drying (data not shown).

Comparison of on-site and laboratory protocols. The performance of the on-site testing method was compared with that of the protocol used at CSL for routine molecular testing for *P. ramorum*. The DNA extraction method used in the laboratory typically yields highly concentrated DNA from cultures and plant material, so a modified *P. ramorum* forward primer with attenuated sensitivity (*Pram*-114Fc; Table 1) is used to rule out the possibility of cross reactions with *P. lateralis*. Eighty randomly selected routine diagnostic samples were tested with both the on-site protocol (modified PickPen extraction followed by multiplex testing on the SmartCycler) and the routine laboratory protocol [NucleoSpin plant kit extraction, using the manufacturer's protocol for fungi, followed by testing on the ABI Prism 7700 for *P. ramorum* (primers 190R and 114Fc) and COX and concurrent isolation on P_5ARP-(H)]. All extracts

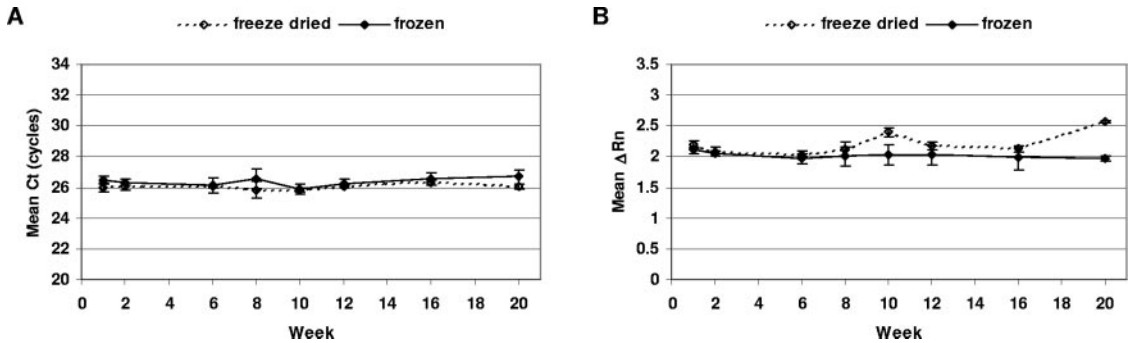

FIG. 4. Long-term storage of freeze-dried real-time PCR reagents. Aliquots of master mix containing *P. ramorum* primers and probe and 5% trehalose were either frozen at −20°C or freeze-dried and then stored at room temperature in the dark and were tested with aliquots of the same extract of DNA from a *P. ramorum*-inoculated rhododendron. (A) C_T values for up to 20 weeks after the preparation of master mix. (B) End-point fluorescence (ΔR_n) values for up to 20 weeks after the preparation of master mix. The C_T and ΔR_n values shown are mean values for six replicate reactions; error bars show standard deviations.

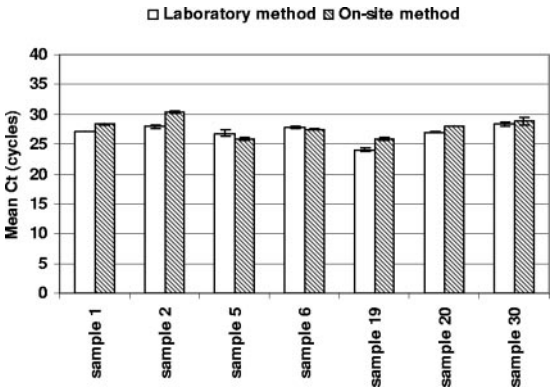

FIG. 5. Comparison of laboratory and on-site real-time PCR methods for detection of *P. ramorum*. Duplicate samples were tested using the laboratory method (DNA extraction using a NucleoSpin plant DNA kit followed by testing on the ABI Prism 7700) and the on-site method (DNA extraction using a modified PickPen extraction method followed by testing on the SmartCycler). The figure shows results for positive samples only. Mean *P. ramorum* C_T values are for duplicate reactions, and error bars show standard deviations.

were tested in duplicate reactions, and mean C_T values were calculated. Seven samples tested positive for *P. ramorum* by isolation and both real-time PCR methods, with C_T values in the range of 25.90 to 30.41 for the on-site method and 24.02 to 28.36 for the laboratory method (Fig. 5). Of the remaining 73 samples, 72 tested negative for *P. ramorum* using both methods (Fig. 6), and a *Phytophthora* species other than *P. ramorum* was isolated from 5 of these samples (7%). One sample tested negative for *P. ramorum* using the laboratory method but gave a *P. ramorum* C_T value of 38.64 when tested using the on-site method. Since neither *P. ramorum* nor any other species of *Phytophthora* was isolated from this sample, it was assumed that this result was most likely due to cross-contamination during extraction.

Testing in the field. The on-site detection method has been tested a number of times in the field, including at an outbreak site in South Wales in November 2004. On this occasion, testing was carried out in a workshop which had a mains electricity supply but no other amenities. One DNA extract was prepared for each sample and tested in multiplex reactions for *P. ramorum* and COX. DNAs were successfully extracted from 33 of 37 samples tested, and *P. ramorum* was detected in 7 samples from five different hosts (*R. ponticum*, *Syringa* sp., *Parrotia persica*, and two *Magnolia* spp.), using freeze-dried multiplex reagents. For five of the *P. ramorum*-positive samples, *P. ramorum* C_T values were in the range of 27.74 to 31.51, with the remaining two positive samples giving C_T values of >36. *P. ramorum* was subsequently isolated from six of the samples which tested positive in the field (the remaining sample deteriorated in transit to the laboratory and could not be tested), including the two samples with C_T values over 36. The on-site testing method has also been used in the absence of mains electricity, using a generator to power the SmartCycler and heating block, near CSL in February 2004, where DNAs were extracted from samples of healthy plant material and tested for COX, and at China Camp State Park, California, in March 2004, where symptomatic and asymptomatic samples from a range of *P. ramorum* host species were tested for *P. ramorum* and COX (data not shown). On both occasions, DNA extraction was carried out in the front seat and reactions were set up in the back seat of the vehicle used for transportation to the testing site.

	Laboratory method (real-time PCR and isolation)			
		+ve	-ve	Total
On-site method (Smart Cycler)	+ve	7 (*A*)	1 (*B*)	8
	-ve	0 (*C*)	72 (*D*)	72
	Total	7	73	80

FIG. 6. Comparison of laboratory and on-site testing for detection of *P. ramorum*. The figure shows positive (+ve) and negative (−ve) results for 80 samples tested for *P. ramorum* using the laboratory and on-site methods, illustrating the diagnostic sensitivity [A/(A + C)] and specificity [D/(D + B)]. The diagnostic sensitivity = 100%, and the diagnostic specificity = 98.6%.

DISCUSSION

The method described here allows a rapid and reliable diagnosis of *P. ramorum* to be made at the point of sampling in under 2 h and gives comparable results to testing by single-round real-time PCR in the laboratory. The sensitivity and specificity of the assay have been shown to be suitable for the detection of *P. ramorum* in DNAs extracted from symptomatic foliage and stems using the on-site method. It should be pointed out, however, that if a different extraction method yielding more concentrated DNA were to be used or if cultures were to be tested rather than plant material, it would be necessary to reassess the specificity and sensitivity of the assay to ensure its suitability for use under these circumstances. At CSL, for example, where a highly efficient method is routinely used for extracting DNA from cultures and plant material in the laboratory, an alternative *P. ramorum* forward primer with attenuated sensitivity (*Pram*-114Fc) was designed to ensure appropriate sensitivity for the extracts to be tested and to rule out any possibility of cross reactions with *P. lateralis*.

The COX internal control assay is used in multiplex mixtures with the *P. ramorum* assay to verify the success of DNA extraction and to identify any false-negative results due to failed extraction. The simplicity of the DNA extraction method, the minimal handling required when using prepared freeze-dried master mix, and the use of a closed-tube, single-round PCR assay all help to minimize the possibility of false-positive results caused by cross-contamination. A number of simple precautions (for example, performing DNA extraction and PCR setup in spatially separate areas, changing gloves frequently and between samples, and predividing extraction buffers into aliquots in the laboratory) further reduce this risk without increasing the length or complexity of the method. The majority of infected samples tested were found to give *P. ramorum* C_T values of <35. C_T values above 35 may be regarded as ambiguous, as they may represent, for example, infection with a low level of pathogen, a sample in poor condition or containing a nonviable pathogen (14), or surface contamination

without infection, as well as possible cross-contamination during handling. Under some circumstances, such a result could warrant further investigation, either in the field (resampling or re-extraction) or at a laboratory facility (molecular testing or isolation). Since the real-time PCR assay does not give a clear indication of pathogen viability, particular care may be necessary in some circumstances, for example, if pesticides have been used in order to suppress symptoms. It is also possible that a high *P. ramorum* C_T value could indicate the presence of a high concentration of *P. lateralis* DNA. This is unlikely, however, because the on-site DNA extraction method typically extracts less DNA than is needed for the amplification of *P. lateralis* (at least 1 ng *P. lateralis* DNA). Unlike *P. ramorum*, *P. lateralis* is a root pathogen, and indeed, it was not found to cause lesions when inoculated onto wounded rhododendron leaves. While *P. lateralis* may have occasionally been isolated from rhododendron tissue with dieback symptoms (8), it is predominantly confined to two hosts (*Chamaecyparis lawsoniana* and *Taxus brevifolia*) (13, 14, 24, 36) which are currently not thought to be affected by *P. ramorum*. It is therefore unlikely that material sampled for *P. ramorum* testing in the field would contain sufficient *P. lateralis* DNA to cause a positive result by the DNA extraction and real-time PCR methods described here.

The stabilization of PCR reagents by freeze-drying allows their unrefrigerated storage and transportation and further simplifies their use in the field. Trehalose is a PCR additive recommended by Cepheid to improve the efficiency and productivity of amplification on the SmartCycler and is also commonly used as a stabilizer for freeze-drying, including freeze-drying of conventional PCR reagents for ambient storage (17). In this study, the addition of trehalose at a final concentration of 5% (wt/vol) was found to improve both C_T values and end-point fluorescence and allowed the master mix to be prepared in advance and stored at room temperature. The quality of batches of master mix can be verified in the laboratory before on-site use, and no decline in performance was observed for up to 5 months after preparation. PCR beads which are stable at room temperature are commercially available, such as puRe*Taq* Ready-To-Go PCR beads (Amersham Biosciences, Little Chalfont, England). Such products have the advantage of not containing a passive reference dye (unlike Applied Biosystems TaqMan core reagents), which is necessary for signal normalization on some real-time PCR platforms, such as the ABI Prism 7700, but is not required for use on the SmartCycler. The performance of the freeze-dried master mix was compared with that of puRe*Taq* Ready-To-Go PCR beads using the *P. ramorum* and COX assays. Lower C_T values were observed for reactions containing the freeze-dried master mix than for those containing puRe*Taq* beads, although the performance of the puRe*Taq* beads was greatly improved by increasing the final concentration of magnesium chloride to 5.5 mM (data not shown). Standard real-time PCR reagents made up into complete master mix and freeze-dried in-house represent an extremely cost-effective, flexible, and reliable alternative to commercially available stabilized PCR reagents.

The *P. ramorum* assay was developed using an ABI Prism 7700/7900HT real-time PCR platform but was adapted for on-site use on a SmartCycler machine, and the potential exists for assays for the detection of a wide range of other plant pathogens to be similarly adapted for use in the field. The generic cycling conditions described here can be used for the detection of a wide range of pathogens by TaqMan PCR, without the need for optimization. However, the optimization of PCR conditions for individual assays and the use of real-time PCR reagents specifically designed for use with rapid-cycling thermal cyclers could further decrease the length of testing to considerably less than 1 hour. The DNA extraction method was also developed from a laboratory-based protocol to make it suitably simple, rapid, and robust for use in the field and has the potential to be used for a range of pathogens in a wide range of hosts. The ability to test plant samples for *P. ramorum* or other pathogens rapidly and at the point of sampling is likely to have a number of useful applications. Epidemiological studies in the field or at remote locations, for example, could greatly benefit from the ability to perform molecular testing without the need to return samples to a laboratory. Also, decisions regarding control or eradication measures, which may need to be taken rapidly, could be better informed by the availability of reliable real-time PCR data on-site and within 2 hours of inspection.

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