Development of a One-Step Real-Time Polymerase Chain Reaction Assay for Diagnosis of *Phytophthora ramorum*

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Accepted for publication 1 April 2006.

ABSTRACT

Hughes, K. J. D., Tomlinson, J. A., Griffin, R. L., Boonham, N., Inman, A. J., and Lane, C. R. 2006. Development of a one-step real-time polymerase chain reaction assay for diagnosis of *Phytophthora ramorum*. *Phytopathology* 96:975-981.

Phytophthora ramorum is a recently described pathogen causing bleeding cankers, dieback, and leaf blight on trees and shrubs in parts of Europe and North America, where the disease is commonly known as sudden oak death. This article describes the development of a single-round real-time polymerase chain reaction (PCR) assay based on TaqMan chemistry, designed within the internal transcribed spacer 1 region of the nuclear ribosomal (nr)RNA gene for detection of *P. ramorum* in plant material. Unlike previously described methods for the molecular detection of *P. ramorum*, this assay involves no post amplification steps or multiple rounds of PCR. The assay was found to have a limit of detection

of 10 pg of *P. ramorum* DNA, and could detect *P. ramorum* in plant material containing 1% infected material by weight within 36 cycles of PCR. The assay also was used to test DNA from 28 other *Phytophthora* spp. to establish its specificity for *P. ramorum*. A quick and simple method was used to extract DNA directly from host plant material, and detection of *P. ramorum* was carried out in multiplex with an assay for a gene from the host plant in order to demonstrate whether amplifiable DNA had been extracted. Amplifiable DNA was extracted from 84.4% of samples, as demonstrated by amplification of host plant DNA. The real-time protocol was used to test 320 plant samples (from 19 different plant species) from which DNA extraction had been successful, and was shown to give results comparable with a traditional isolation technique for diagnosis of *P. ramorum* in plant material from common U.K. hosts.

Additional keywords: rhododendron, viburnum.

In the United States, *Phytophthora ramorum* causes mortality of native American oak trees (*Lithocarpus densiflorus* and *Quercus* spp.) in woodlands on the west coast (24). This pathogen also causes dieback or leaf blight on a wide range of other woodland trees, shrubs, and several herbaceous plant species and has been found in nurseries in a number of states (6). In Europe, it is recorded mainly in nurseries, where the most commonly reported hosts are rhododendron (18,34), viburnum (19), and camellia (2). However, a wider range of other host species has been reported which includes *Hamamelis virginiana* (11), *Pieris formosa* (14), *Syringa vulgaris* (3), and *Taxus baccata* (20). *Phytophthora ramorum* also has been recorded on several native and nonnative tree species in the United Kingdom, causing either bleeding bark cankers (e.g., *Aesculus hippocastanea*, *Fagus sylvatica*, *Q. cerris*, *Q. falcata*, and *Q. rubra*) or leaf and shoot infections (e.g., *Castanea sativa* and *Q. ilex*) (4,5). In both the United States and Europe, *P. ramorum* is considered an exotic pathogen which has been introduced separately on each continent (15,26). European isolates and North American woodland isolates generally represent two distinct populations (15). Isolates from the European population are almost all of the A1 mating type, although a single European A2 isolate is known from Belgium (32); isolates from North America all have been reported as the A2 mating type, except for isolated outbreaks in nurseries in Oregon, Washington State, and British Columbia (26). Due to the potential threat to trees, woodlands and heathlands, emergency European Commission phytosanitary measures were implemented in November 2002 to prevent the introduction and spread of *P. ramorum* within the European Union (1). Therefore, development of reliable,

accurate, and rapid diagnostic methods for symptomatic tissue became essential. Diagnosis of *P. ramorum* can be performed using traditional mycological methods involving isolation from host material followed by morphological identification with microscopy in comparison with a published description of the organism (33). However, this relies on key taxonomic features being present and skilled mycologists to differentiate *P. ramorum* from the potential range of other *Phytophthora* spp.; if these taxonomic features are absent, overlooked, or misidentified, this may lead to an incorrect diagnosis. Additionally, successful isolation may be dependent on factors such as the time of year (7). Diagnosis using conventional polymerase chain reaction (PCR) can support traditional morphological approaches, and such methods have been developed for *P. ramorum* using primers designed for the internal transcribed spacer (ITS) 1 and 2 regions of the nuclear ribosomal gene for *P. ramorum* (10,18) and subunit 1 and 2 of the mitochondrial cytochrome oxidase gene (22). Gel-based molecular detection methods such as single-strand conformation polymorphism (SSCP) analysis (17) and restriction fragment length polymorphism (RFLP) analysis (21) also can be used to identify *P. ramorum* from other *Phytophthora* spp., but such systems can be difficult to interpret and are not suited to the high-throughput testing that is necessary for national surveys.

Real-time PCR assays (27), especially those based on TaqMan chemistry, are becoming increasingly preferred to gel-based PCR because they are quicker, generally offer increased specificity and sensitivity, and are performed in a closed tube system which is less prone to post-PCR contamination (9,23,30). TaqMan assays amplify DNA in the same way as conventional PCR but exploit a dual-labeled fluorescent probe (designed to hybridize to the DNA sequence between the forward and reverse primers) and the 5'-3' exonuclease activity of Taq polymerase to produce a fluorescent signal. As amplification occurs, the probe is cleaved in a sequence-specific fashion resulting in an increase in fluorescence propor-

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DOI: 10.1094/PHYTO-96-0975

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TABLE 1. Results following real-time polymerase chain reaction testing of a range of *Phytophthora* isolates with primers designed to the internal transcribed spacer 5.8S region and to *P. ramorum*^a

Species tested	Reference	Ct value, amplification using		
		5.8S primers	Pram-114F	Pram-114Fc
<i>P. boehmeriae</i>	P 6950 ^b	16.28	–	–
<i>P. boehmeriae</i>	CBS 100410 ^c	16.56	–	–
<i>P. botryosa</i>	P 6945 ^b	18.04	–	–
<i>P. cactorum</i>	MUCL9638 ^d	17.93	–	–
<i>P. cactorum</i>	CSL 2151 ^e	18.09	–	–
<i>P. cambivora</i>	CBS 376.61 ^c	16.60	–	–
<i>P. cambivora</i>	FR P315 ^f	17.06	–	–
<i>P. cinnamomi</i>	SCRI CIN5 ^g	16.03	–	–
<i>P. cinnamomi</i>	PD 93/1389 ^h	16.23	–	–
<i>P. citricola</i>	CSL 1531 ^e	16.25	–	–
<i>P. citricola</i>	CSL 2098 ^e	16.43	–	–
<i>P. citricola</i>	SCRI CIT1 ^g	16.86	–	–
<i>P. citricola</i>	CSL 1530 ^e	17.07	–	–
<i>P. citricola</i>	CBS379.61 ^c	17.14	–	–
<i>P. citrophthora</i>	IMI 132217 ⁱ	16.07	–	–
<i>P. cryptogea</i>	SCRI P521 ^g	16.95	–	–
<i>P. erythrosetpica</i>	P 7889 ^b	18.02	–	–
<i>P. europaea</i>	CBS 109053 ^c	17.39	–	–
<i>P. fragariae</i> var. <i>rubi</i>	FR-1631 ^j	16.77	–	–
<i>P. gonopodyides</i>	P 10337 ^b	16.28	–	–
<i>P. heveae</i>	CBS 958.87 ^c	17.04	–	–
<i>P. hibernalis</i>	P 3822 ^b	17.45	–	–
<i>P. ilicis</i>	P 3939 ^b	17.37	–	–
<i>P. insolita</i>	P 6195 ^b	17.65	–	–
<i>P. kernoviae</i>	FR CAE36B ^f	16.94	–	–
<i>P. kernoviae</i>	CSL 2169 ^e	16.98	–	–
<i>P. kernoviae</i>	CSL 2306 ^e	17.15	–	–
<i>P. kernoviae</i>	FR CAE4 ^f	17.61	–	–
<i>P. kernoviae</i>	FR P1560 ^f	17.88	–	–
<i>P. lateralis</i>	P 3888 ^b	18.69	32.55	–
<i>P. lateralis</i>	P 1728 ^b	16.96	31.07	38.95
<i>P. macrochlamydospora</i>	P 10263 ^b	17.10	–	–
<i>P. megasperma</i>	CBS 320.49 ^c	16.26	–	–
<i>P. nemorosa</i>	P 10288 ^b	17.18	–	–
<i>P. nicotianae</i>	CBS 411.87 ^c	17.72	–	–
<i>P. nicotianae</i>	SCRI NIC1 ^g	16.48	–	–
<i>P. palmivora</i>	SCRI P488 ^g	17.50	–	–
<i>P. pseudosyringae</i>	CSL2369 ^e	15.79	–	–
<i>P. pseudosyringae</i>	P 10444 ^b	16.15	–	–
<i>P. quercina</i>	P 10334 ^b	17.10	–	–
<i>P. ramorum</i> (Germany)	BBA 69082 ^k	18.51	15.79	22.44
<i>P. ramorum</i> (Germany)	BBA 14/98a ^k	18.04	15.36	22.11
<i>P. ramorum</i> (U.K.)	CSL 1684 ^e	17.55	15.37	22.26
<i>P. ramorum</i> (U.K.)	CSL 1667 ^e	19.73	16.94	24.07
<i>P. ramorum</i> (U.S.A.)	P1349 ^k	17.94	15.35	21.96
<i>P. ramorum</i> (U.S.A.)	P1403 ^l	19.27	16.33	22.81
<i>P. ramorum</i> (U.S.A.)	P1347 ^f	19.54	16.55	23.90
<i>P. ramorum</i> EU A1 ^m	P1376 ^f	17.22	15.12	21.33
<i>P. ramorum</i> US A2 ⁿ	P1579 ^h	17.79	16.32	23.21
<i>P. richardiae</i>	P 7788 ^b	17.12	–	–
<i>P. richardiae</i>	P 10335 ^b	17.77	–	–
<i>P. richardiae</i>	P 7789 ^b	17.80	–	–
<i>P. syringae</i>	CBS 272.55 ^c	16.34	–	–
<i>P. syringae</i>	4N0247-5 ^j	16.53	–	–
<i>P. syringae</i>	4N0247-6 ^j	16.93	–	–
<i>P. syringae</i>	CBS 364.52 ^c	17.84	–	–
<i>P. uliginosa</i>	CBS 109055 ^c	17.25	–	–

^a Country of isolation for *P. ramorum* is indicated where known. Cycle threshold (Ct) values are mean values for duplicate reactions; – indicates a negative result.

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^c CBS, Utrecht, The Netherlands.

^d Belgian Co-ordinated Collections of Micro-organisms.

^e Central Science Laboratory, York, U.K.

^f Forest Research, Alice Holt, Farnham, U.K.

^g D. Cooke, SCRI, U.K.

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^j Laboratoire National de la Protection des Végétaux, Nancy, France.

^k S. Werres, BBA, Braunschweig, Germany.

^l E. Hansen, University of Oregon.

^m Confirmed as A1 mating strain by Forest Research.

ⁿ Confirmed as A2 mating strain by Forest Research.

tional to the amount of product amplified. The number of cycles it takes for a sample to produce fluorescence exceeding a threshold value is referred to as the cycle threshold (Ct) value and can be used to compare amplification between samples. The ability of real-time machines like the ABI Prism 7900HT (Applied Biosystems, Foster City, CA) to measure fluorescence at various wavelengths simultaneously allows reactions to be multiplexed using different dye labels, allowing internal control assays to be combined with target-specific assays (31).

A nested real-time PCR assay has been developed for the specific detection of *P. ramorum* (12), based on SYBR green technology. Because the protocol is a nested PCR, an initial PCR step is performed prior to the real-time PCR step. However, because the assay comprises multiple steps, it may be prone to contamination introduced between the first and second round of PCR. Also, because the assay is based on SYBR green dye, it is necessary to perform melting profile analysis to ascertain whether amplification was specific or due to accumulation of primer dimers or nonspecific amplification products. This article describes a streamlined, single-round real-time PCR assay for the specific detection of *P. ramorum*. A simple procedure was used for extraction of DNA from cultures and host plant material which, unlike some previously described methods, does not require use of organic solvents or pre-freezing or lyophilization of tissue, making it potentially suitable for use in a routine diagnostic setting. *P. ramorum*-specific primers and probe were used in multiplex with generic plant cytochrome oxidase (COX) primers and probe to allow detection of host plant DNA, helping to safeguard against false-negative results. Of 379 samples submitted for testing, DNA was successfully extracted from 320 samples (84.4%) of 19 different plant species. The 320 samples were tested for *P. ramorum* using the real-time detection method and the results were found to compare favorably with isolation and morphological identification.

MATERIALS AND METHODS

Maintenance of isolates. In all, 57 isolates from 29 different *Phytophthora* spp. were obtained from around the world (Table 1). The species name provided by the isolate supplier was adopted as the isolate's identity and isolates were maintained on carrot piece agar (CPA) (33) at $\approx 20^{\circ}\text{C}$ under 12 h of light and 12 h of dark, or underwater at 4°C in the Central Science Laboratory (CSL) culture collection until required. Some isolates also were grown for 2 weeks on 4% potato dextrose agar (PDA) at $\approx 20^{\circ}\text{C}$ under 12 h of light and 12 h of dark to obtain DNA for sequencing. Prior to use, samples of each batch of CPA were subjected to the DNA extraction protocol described below and tested using the 5.8S real-time PCR assay to ensure the absence of any amplifiable *Phytophthora* DNA within the medium.

Extraction of DNA from cultured isolates. DNA extraction from cultures was performed using a NucleoSpin Plant extraction kit (Macherey-Nagel, Düren, Germany). Each isolate was grown for 2 weeks on CPA; then, 1-cm² plugs were taken from each isolate, avoiding pieces of carrot within the agar. Each plug was placed in a 2-ml microcentrifuge tube; then, a similar volume of sterile 0.5-mm glass beads (BioSpec Products Inc., Bartlesville, OK) was added, along with 300 μl of NucleoSpin C1 buffer. Tubes were sealed with a screw-cap lid containing an o-ring and shaken in a MiniBeadbeater-8 homogenizer (BioSpec Products Inc.) for 3 min on quarter power to macerate the sample. DNA extraction was completed following the NucleoSpin Plant kit support protocol for fungi. Where relevant, DNA was quantified spectrophotometrically.

ITS sequencing. *Phytophthora* isolates were grown for 2 weeks on 4% PDA as described; then, DNA was extracted using the NucleoSpin method and the ITS and 5.8S regions were amplified using PCR, as described previously (13), and sequenced. The following accessions were deposited in GenBank: *P. citricola*

isolates CSL cc1530/P920 (AY769941) and CSL cc1531/P1013 (AY769942); *P. kernoviae* isolate CSL cc2124 (DQ002008); *P. lateralis* isolate CSL cc2090/CBS168.42 (AY785952); and *P. ramorum* isolates CSL cc1527/P1367 (AY785958), CSL cc1528/P1347 (AY785959), and CSL cc1529/P1348 (AY785960).

TaqMan probe and primer design. Multiple sequence alignments were performed on new sequences and sequences from GenBank using the Clustal V method from the MegAlign program (DNASTar Inc., Madison, WI). Probes and primers for *P. ramorum* were designed within the most divergent sequence regions identified among species in the ITS 1 region of the nuclear ribosomal (nr)RNA gene (Fig. 1). The probe and primer combinations were designed using Primer Express software (Applied Biosystems, Foster City, CA) and modifications to these designs subsequently were introduced with the intention of improving discrimination between *P. ramorum* and other *Phytophthora* spp. with similar sequences (25,29) (Fig. 1). Primer and probe sequences, and probe reporter and quencher dyes, are shown in Table 2. Primers and a TaqMan probe also were designed within the 5.8S region of the nuclear ribosomal (nr)RNA gene (Table 2) from several *Phytophthora* spp. (alignment not shown). These acted as a control assay showing that amplifiable DNA was extracted from all *Phytophthora* isolates tested. Plant internal control primers and probe were based on a previously described assay designed in a conserved region of the plant COX gene (31). Primers were synthesized by TagN (Newcastle, England) and probes were synthesized by Applied Biosystems.

Real-time PCR. Real-time PCRs were set up in 96-well reaction plates using TaqMan Universal PCR Master Mix or TaqMan

Core Reagents (Applied Biosystems) consisting of 1× buffer A (50 mM KCl, 10 mM Tris-HCl, pH 8.3, ROX passive reference dye) and AmpliTaq Gold at 0.025 U/μl, plus 0.2 mM each dNTP and 5.5 mM MgCl₂. For each reaction, 1 μl of DNA extract was added, giving a final volume of 25 μl, and negative controls containing nuclease-free water instead of DNA were included in every run. When testing DNA extracted from cultures, reactions contained either *P. ramorum* or 5.8S primers and probe at final concentrations of 300 nM for each primer and 100 nM for the probe. When testing DNA extracted from plant material, multiplex reactions containing *P. ramorum* primers (300 nM each) and probe (100 nM) and COX primers (200 nM each) and probe (100 nM) were used. Real-time PCR was carried out on an ABI Prism 7900HT (Applied Biosystems) using generic cycling conditions (50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min). The Ct value for each reaction was assessed using the Sequence Detection Software's default threshold setting of 0.2 ΔRn (fluorescence) units.

Production of artificially infected plant material. Detached healthy leaves and stems of *Rhododendron ponticum* and *Viburnum tinus* and leaves of a *Camellia* sp. were wounded and inoculated with 0.5-cm² plugs taken from the margin of 7-day-old cultures of *P. ramorum* on CPA (CSL isolate ref. 1604). Inoculated material then was incubated in a damp chamber at room temperature for 7 days to initiate infection.

Extraction of DNA from plant material. DNA was extracted from artificially and naturally infected plant material. Weighed samples (0.2 to 0.5 g) were placed in extraction bags (Bioreba, Reinach, Switzerland) with 8 to 10 volumes of NucleoSpin C1

Fig. 1. Multiple sequence alignment for a range of *Phytophthora* spp. to which the *Phytophthora ramorum*-specific assay was designed. Boxes identify the position of the primers and probe; · indicates nucleotides identical to the *P. ramorum* sequence and – indicates nucleotides not present. The 5' and 3' nucleotides for each of the sequences in the alignment are as follows, AY785958 (104–171), AF266804 (93–160), AF266770 (86–151), L41357 (100–166), L41375 (135–200), AF228086 (128–196), AF087478 (145–219), AF139369 (115–190), and AJ007040 (145–220). Additional sequences compared but not included in the figure were *P. ramorum* (AY785959, AY785960, AY038050, AY785955, and AY785956), *P. boehmeriae* (AF228076), *P. cactorum* (AF087480, AF228077, and AF266772), *P. cambivora* 1 (AF087479), *P. cambivora* 2 (AF266763); *P. cinnamomi* (AF266764), *P. citricola* (AY769942, AY785954, and AY769941), *P. idaei* (CSL 1366), *P. lateralis* (AF287256), *P. nemorosa* (AY332654), *P. nicotianae* (AF228086, AF228085, and AF228085), *P. pseudosyringae* (AY366462 and AY366463), and *P. kernoviae* (CSL 2124).

TABLE 2. Characteristics of primers and TaqMan probes^a

Primer or probe	Sequence (5'–3')	Position of 5' base in sequence	Reporter (5') ^b	Quencher (3') ^b	Final concentration (nM)
<i>Pram</i> -114F	TCATGGCGAGCGCTTGA	104	...	...	300
<i>Pram</i> -114Fc	TCATGGCGAGCGCTGGA	104	...	...	300
<i>Pram</i> -190R	AGTATATTTCAGTATTTAGGAATGGGTTTAAAAAGT	180	...	...	300
<i>Pram</i> probe	TTCGGGTCTGAGCTAGTAG	124	FAM	TAMRA	100
COX F	CGTCGCATTCCAGATTATCCA	1,488	...	...	200
COX R _w	CAACTACGGATATATAAGRRCCRAACTG	1,566	...	...	200
COX probe	AGGGCATTCCATCCAGCGTAAGCA	1,534	VIC	TAMRA	100
5.8S F	TGTCTAGGCTCGCACATCGA	232	...	...	300
5.8S R	GATGACTCACTGAATCCTGCAATT	308	...	...	300
5.8S probe	ACGCTGCGAACTGCGATACGTAATGC	258	JOE	BHQ1	100

^a Table shows the sequence and position of the primers and probes and fluorescent dyes used. The position of the 5' bases of the forward and reverse primers and the probes are relative to the sequence accession AY785958 for the *Phytophthora ramorum* and 5.8S assays, and in the sequence accession X83206 for the cytochrome oxidase (COX) internal control assay.

^b FAM = 6-carboxyfluorescein, TAMRA = tetra-methylcarboxyrhodamine, VIC = Applied Biosystems, Foster City, CA, JOE = 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein, and BHQ1 = Black Hole Quencher 1, Biosearch Technologies, Novato, CA.

buffer. Each sample then was homogenized using a Homex grinder (Bioreba) and DNA was extracted from 300- μ l aliquots using the NucleoSpin method described above. For the comparative testing of symptomatic plant material, samples of ≈ 0.2 g wet weight were ground in 1.6 ml of Nucleospin C1 buffer.

Isolation of *Phytophthora* spp. from plant material. Plant material (leaf or stem) with suspected symptoms was collected by Defra's Plant Health and Seeds Inspectorate (PHSI) and placed in a sealed plastic bag with a small piece of damp tissue prior to dispatch from the field. On receipt in the laboratory, samples were examined for the presence of typical lesions; small pieces of tissue were aseptically excised from the leading edge of the lesions. These were placed in a small plastic bag with ≈ 50 ml of distilled water for ≈ 1 h to aid rehydration, during which time the bags were shaken vigorously for 15 to 20 s to remove any debris. At the end of the hour, the water was changed twice to remove debris that had been dislodged by the washing process. The tissue then was plated out onto a semiselective agar (P_5 ARP(H)) (16) and the plates were examined microscopically after 6 days incubation ($\approx 20^\circ\text{C}$, 12 h of light and 12 h of dark) for the presence of any *Phytophthora* sp. and, specifically, *P. ramorum* growth.

RESULTS

TaqMan probe and primer design. Primers and a TaqMan probe were designed (Table 2) to the most species-specific region identified in the ITS 1 region following a multiple sequence alignment between *P. ramorum* and some of the most closely related *Phytophthora* spp. (Fig. 1). In addition to primer *Pram*-114F, which matched the sequence of *P. ramorum*, a second forward primer was designed (*Pram*-114Fc) which included a base substitution at the third base from the 3' end (guanine instead of thymine) in order to make the primer sequence more distinct from the ITS target sequence of closely related species. A real-time assay also was designed within the conserved region of the 5.8S sequence of the nrRNA gene to allow the detection of DNA for all *Phytophthora* spp. tested.

Assay specificity. In all, 57 isolates of 29 different species of *Phytophthora* were tested using the *P. ramorum* real-time assay and the 5.8S control assay (Table 1). Results of the 5.8S assay

showed that all extracts contained amplifiable DNA, with Ct values varying between 16.03 and 19.73 (estimated using a standard curve as corresponding to ≈ 10 to 100 ng; data not shown). *P. ramorum* isolates gave Ct values between 15.12 and 16.94 when tested with the *P. ramorum* assay incorporating the *Pram* 114F primer, and between 21.33 and 24.07 when tested using the *Pram*-114Fc primer. All other *Phytophthora* isolates tested negative with both *P. ramorum* primer sets, with the exception of *P. lateralis* (Table 1). The two isolates of *P. lateralis* gave mean Ct values of 32.55 and 31.07 when tested with primer *Pram*-114F, and one isolate (ref P1728) gave a mean Ct value of 38.95 when tested with primer *Pram*-114Fc (the other *P. lateralis* extract was not amplified when tested using this primer). Despite giving higher Ct values for *P. ramorum* isolates, *Pram*-114Fc was chosen for completion of the comparative testing, due to the higher Ct values observed when testing *P. lateralis* DNA.

Assay efficiency and limit of detection. A dilution series of DNA extracted from *P. ramorum* culture was tested using the TaqMan assay incorporating forward primer 114Fc. The response of the assay was linear down to 10 pg (Fig. 2), and the efficiency of amplification (calculated from the slope of the standard curve as equal to $10^{(-1/\text{slope})} - 1$) was 0.98. The *P. ramorum* assay incorporating primer 114F, and the 5.8S assay, were calculated to have very similar efficiencies when tested with the same dilution series (data not shown). In order to determine whether DNA extracted from rhododendron as described above contains high levels of inhibitors, such as polyphenolics, which could adversely affect sensitivity, the dilutions also were tested with 1 μ l of DNA extracted from healthy rhododendron leaf added to each reaction (Fig. 2). The rhododendron extract contained DNA at ≈ 5 ng/ μ l, an amount typically observed for extracts from rhododendron leaf. A slight decrease in the efficiency of the assay was observed in the presence of rhododendron extract, calculated from the standard curve as above (0.88 compared with 0.98 for *P. ramorum* DNA alone); however, the Ct values were very similar over the range of *P. ramorum* DNA concentrations tested in the presence and absence of rhododendron DNA (Fig. 2). Amplification of *P. ramorum* DNA at lower concentrations became somewhat more variable and did not fall within the linear range of the assay. However, more dilute samples did give positive results; the lowest amount of DNA amplified was 5 pg in both the presence and absence of rhododendron DNA (Ct values 37.87 ± 0.57 and 37.78 ± 0.66 , respectively). The dilutions of *P. ramorum* DNA also were tested in multiplex for *P. ramorum* and COX in the presence of 5 ng of rhododendron DNA per reaction and, in this case, the lowest amount of *P. ramorum* DNA amplified was 10 pg (mean Ct value 38.49 ± 0.62); the efficiency was somewhat reduced (the slope of the standard curve was -4.10 , giving an efficiency of 0.75; the R^2 value for the curve was 0.998).

The sensitivity of the assay was also assessed in terms of its ability to detect *P. ramorum* in infected plant material. Necrotic tissue from *P. ramorum*-inoculated detached leaves/stem of *R. ponticum* (leaf and stem), *V. tinus* (leaf and stem), and *Camellia* sp. (leaf) was mixed with healthy material of the same host species and tissue type to produce samples with a total wet weight of 0.5 g, containing 0, 10, 5, and 1% necrotic tissue by weight. Samples were ground in 5 ml of NucleoSpin C1 buffer, and for each species and tissue type, the ground sap containing 1% infected material was diluted in ground healthy sap to produce samples containing 0.1% infected material. DNA was extracted from two 300- μ l subsamples of each dilution and tested using the *P. ramorum* assay based on the *Pram*-114Fc primer in multiplex with the COX internal control assay. COX Ct values ranged between 21.31 (± 0.78) for *R. ponticum* stem and 24.77 (± 1.51) for *V. tinus* leaf, indicating that the amount of amplifiable DNA that was extracted from the five host/tissue types used fell within an approximately 10-fold range. In each host and tissue type tested, *P. ramorum* could be detected down to 0.1% infected

Fig. 2. Linear relationship between DNA concentration and cycle threshold (Ct) value for *Phytophthora ramorum* DNA amplified using TaqMan primers and probe. A dilution series of DNA extracted from *P. ramorum* culture was tested by real-time polymerase chain reaction using the *P. ramorum* assay (primers *Pram*-114Fc and 190R). The same dilutions also were tested with the addition of ≈ 5 ng of DNA extracted from healthy rhododendron leaf per 25 μ l of reaction. Ct values shown are mean values for three replicate reactions; error bars show standard deviations.

material, and all samples containing 1% infected material gave Ct values of <36 cycles, ranging from 33.34 (± 0.59) to 35.64 (± 0.31).

Comparative testing of plant samples. A large number of samples ($n = 379$), collected between October 2003 and February 2004 by the PHSI as part of the national *P. ramorum* survey of England and Wales, were used to perform a comparative test between isolation and TaqMan testing of plant material. Samples were examined for typical symptoms and four small pieces of tissue (≈ 0.4 g total wet weight) were taken from the leading edge of lesions and rehydrated as described. One-half of each piece was plated out onto P₅ARP(H), and DNA was extracted from the remaining 0.2 g of tissue. DNA extracts were tested by multiplex TaqMan PCR using the *P. ramorum* assay (incorporating primer *Pram-114Fc*) and the internal control COX assay. In order to avoid potentially ambiguous results, the real-time data was interpreted conservatively in this experiment using an arbitrary cut-off of 36 (i.e., 10% below 40 cycles); that is, a mean Ct value (for duplicate reactions) of <36 was regarded as positive whereas a mean Ct value of >36 was regarded as negative (in practice, only five samples gave mean Ct values above 36).

Of the 379 samples tested, amplifiable DNA was not extracted from 59 samples, as indicated by a negative internal control (COX) result. These samples were representative of the range of plant taxa tested; no plant species were encountered from which we were consistently unable to recover DNA. Such a result indicates that initial testing has been inconclusive and that further testing (re-extraction or resampling) is required in order to obtain a valid real-time PCR result. However, it was not possible to carry out re-extraction as part of this survey; therefore, because testing was incomplete for these samples, they were considered separately from the 320 samples from which amplifiable DNA was obtained. *P. ramorum* was isolated from 1 of the 59 COX-negative samples, and a *Phytophthora* sp. other than *P. ramorum* was isolated from a further 5 of these samples (no *Phytophthora* infection was detected in the remaining 53 samples).

Extraction of DNA, as indicated by amplification with the COX assay, followed by real-time PCR testing was completed for 320 samples. Most of the samples tested were rhododendron (219 samples) and viburnum (54 samples). In addition, DNA was extracted successfully from a further 47 samples from 17 different plant taxa as follows (numbers in parentheses indicate number of samples tested): *Camellia* ($n = 4$), *Corylus* ($n = 4$), *Hamamelis* ($n = 3$), *Hydrangea* ($n = 1$), *Kalmia* ($n = 3$), *Leucothoe* ($n = 1$), *Peronskia* ($n = 1$), *Phygelius* ($n = 1$), *Picea* ($n = 1$), *Pieris* ($n = 11$), *Quercus* ($n = 1$), *Sambucus* ($n = 1$), *Syringa* ($n = 4$), *Taxus* ($n = 6$), *Thuja* ($n = 1$), unknown ($n = 1$), and *Vaccinium* ($n = 3$).

All of the samples on which *P. ramorum* was identified (real-time PCR Ct value <36 or successful isolation) were either rhododendron or viburnum. For these samples, mean *P. ramorum* Ct values were 29.32 ± 3.19 for rhododendron and 27.10 ± 2.47 for viburnum. Results were compared for all 320 samples for which testing was completed: 25 samples gave positive results with both methods, whereas 291 samples gave negative results with both methods. Of the remaining four samples, two (both rhododendron) were negative by real-time PCR but positive by isolation, whereas two (one rhododendron and one viburnum) were negative by isolation but positive by real-time PCR (Table 3). When the diagnostic sensitivity and specificity were calculated for these samples (92.59 and 99.32%, respectively) (Table 3), they showed that the real-time PCR method was highly specific and sensitive, giving few false-positive and false-negative results when the isolation technique was used as a baseline.

Further efforts to isolate *P. ramorum* from the two samples that gave positive results by real-time PCR and negative results by initial isolation revealed that *P. ramorum* was present in both samples. In one case, *P. ramorum* was found by baiting the sample (a desiccated rhododendron shoot) in Petris mineral solution (8)

with a healthy rhododendron leaf bait. For the second (viburnum), *P. ramorum* was identified following extended incubation of the isolation plate. These results suggest that the use of real-time PCR could be beneficial when testing samples in poor condition from which isolation may not be immediately successful; for example, samples collected at certain times of year (7). Further testing of the remaining two samples, which were positive by isolation but negative by PCR, was not possible because insufficient material remained after the initial testing.

Of the 187 rhododendron samples that gave negative results for *P. ramorum*, another *Phytophthora* sp. (most commonly *P. cactorum*) (P. Beales, CSL, U.K., *personal communication*) was detected by isolation from 39 samples (no *Phytophthora* infection was detected in the remaining 148 samples). In each case, these gave negative results using real-time PCR, illustrating the specificity of the primers.

DISCUSSION

This article reports the development and evaluation of a real-time PCR assay based on TaqMan chemistry for the detection of *P. ramorum* directly from common U.K. ornamental hosts, and for specific identification of *P. ramorum* isolated into culture. The assay was subjected to a parallel evaluation alongside a standard isolation technique and was shown to be suitably specific and sensitive for the detection of *P. ramorum* in infected plant material.

Currently, a common technique for detection of *P. ramorum* from plant material is to isolate the pathogen by culturing on semiselective media. This technique is time consuming and results in a delay of 6 to 9 days from when samples are received in the laboratory to when a result is available. Methods based on PCR allow direct detection of the pathogen from plant material; however, methods published to date may not be suited to large-scale use in a routine testing laboratory. Methods based on conventional PCR (10,18) require the time-consuming step of gel electrophoresis and, being open-tube systems, are potentially susceptible to post-PCR contamination. A method for *P. ramorum* detection based on real-time PCR has been published (12) which has a number of advantages over conventional PCR. However, this method relies on a nested-PCR approach which is relatively time consuming and could be prone to contamination problems, and these drawbacks could restrict its use for high-throughput testing. The routine molecular testing of large numbers of samples also requires a rapid and simple DNA extraction method. A method was developed that would enable specific detection of *P. ramorum* in a single round of PCR, making the amplification a closed-tube system, and TaqMan chemistry was employed to enable simple interpretation using Ct values alone. An extraction method was used that allows rapid and simple extraction, without

TABLE 3. Comparison of the real-time polymerase chain reaction (PCR) (TaqMan) method with isolation into culture for the detection of *Phytophthora ramorum*^a

		Culture isolation		Total
		+	–	
Real-time PCR (Taqman)	+	25	2	27
		A	B	
	–	2	291	293
		C	D	
	Total	27	293	320

^a Positive (+) and negative (–) results for 320 samples tested using both methods, illustrating diagnostic sensitivity [$A/(A + C)$] and specificity [$D/(D + B)$]. Samples where DNA extracts were shown to be nonamplifiable (using the internal positive control) were excluded from the analysis. Diagnostic sensitivity = 92.59% and diagnostic specificity = 99.32%.

the use of toxic reagents such as phenol, from up to 50 samples within 2 to 3 h.

The *P. ramorum* assay was designed using new and published sequence data from the ITS 1 region of the nrRNA gene. Because the sequences in this region are very similar for some *Phytophthora* spp., two primer sets were developed, one in which the forward primer (*Pram*-114F) matched the sequence of *P. ramorum* identically, and a second in which a single nucleotide was changed in the forward primer (*Pram*-114Fc), with the intention of enabling better discrimination of species with similar sequences. When concentrated DNA extracts (≈ 10 to 100 ng/ μ l) from a large range of *Phytophthora* spp. were tested using the two primer sets, it was shown that the assay incorporating *Pram*-114F not only amplified DNA from all isolates of *P. ramorum* but also showed cross-reactivity with *P. lateralis* DNA (Ct values 32.55 and 31.07) (Table 1). The assay incorporating primer *Pram*-114F has a limit of detection of ≈ 100 fg of *P. ramorum* DNA (28) compared with a limit of detection of 12.9 fg for the nested real-time PCR assay of Hayden et al. (12), but achieves this within a single round of real-time PCR. Both assays display similar cross-reactivity with high concentrations of *P. lateralis* DNA, both requiring in the region of 1 ng of DNA for amplification (12,28). The primer set chosen for the testing of symptomatic leaf/stem samples in comparison with isolation (*Pram*-114Fc and 190R) detected only one of the two *P. lateralis* extracts tested (Ct value 38.95), indicating that the concentration of these extracts was close to the limit of detection for *P. lateralis*. However, the nucleotide change at the 3' end of this forward primer, which was introduced to achieve increased specificity (25,29), resulted in the assay being somewhat less sensitive than *Pram*-114F, with a limit of detection for purified pathogen DNA of ≈ 5 pg of *P. ramorum* DNA. Sensitivity experiments were performed in order to determine whether the assay incorporating *Pram*-114Fc would be suitable for detection of *P. ramorum* in infected plant material, despite its attenuated sensitivity compared with *Pram*-114F. It was shown that the assay was sufficiently sensitive to allow reliable detection of the pathogen in samples containing as little as 1% symptomatic material by weight when used in conjunction with the Nucleospin DNA extraction method described. In the United Kingdom, the assay is used to confirm the presence of pathogen in samples containing ≈ 25 to 100% symptomatic material, which allows a good safety margin regarding sensitivity when using this DNA extraction method. The assay also detected *P. ramorum* in samples containing 0.1% infected material, with Cts in the range of 36.17 to 38.84. However, a high *P. ramorum* Ct value may be regarded as potentially ambiguous in some circumstances because, instead of indicating a sample containing a low level of *P. ramorum*, such a result could represent a sample in poor condition, surface contamination without genuine infection, cross contamination between extracts, or potentially, a very high concentration of *P. lateralis* DNA (although this is unlikely due to the narrow host range of this pathogen) (12). In practice in a routine testing laboratory, a Ct value between 36 and 40 should be treated as ambiguous and indicative that resampling and testing is required.

The protocol for testing plant material also incorporated an internal control assay designed to detect a gene from the host plant, the use of which aids in the interpretation of negative results, by preventing false negatives. In contrast, no such control of false negative results is available when testing by isolation because, although failure to isolate *P. ramorum* from a sample could genuinely reflect absence of the pathogen, there are a number of factors, such as the condition of the sample, which could adversely affect viability. A negative COX assay result (together with a negative *P. ramorum* result) indicates the need for re-extraction, resampling, or both in order to obtain a conclusive real-time PCR result. Amplifiable DNA was not recovered from 59 of 379 samples submitted for testing by the PHSI, indicating that, although the extraction method was successful for the majority of

samples (84%), modifications to the procedure are likely to be necessary in order to extract successfully from all samples encountered. Samples which are in poor condition (for example, highly desiccated or colonized by other organisms) may contain little intact DNA, or may contain high levels of inhibitors which could adversely affect PCR, which could cause negative COX results. This highlights a need to ensure that care is taken to sample material that will be conducive to successful extraction of amplifiable DNA; for example, taking material from the leading edge of lesions rather than severely necrotic areas. Although the simple, commercially available extraction technique used was found to be successful for extraction of DNA from the majority of samples encountered in the course of survey work in the United Kingdom, in order to take into account the expanding host range of *P. ramorum* and, in particular, the recent findings on tree species in the United Kingdom, it is likely that the DNA extraction method will have to be adapted in line with the material to be tested, particularly if the method is to be used predominantly for detection of *P. ramorum* in forest species rather than ornamental hosts.

The remaining 320 samples from which DNA was extracted successfully were tested in order to compare the *P. ramorum*/COX real-time PCR assay with a traditional isolation technique. A diverse range of hosts was tested, all of which showed dieback or leaf blight similar to the characteristic symptoms of *P. ramorum* infection. Most samples were from rhododendron (68%), but other common natural hosts such as viburnum, camellia, and *Pieris* spp. were included. Less common natural hosts also were encountered, such as *Hamamelis*, *Kalmia*, *Leucothoe*, *Vaccinium*, and *Syringa* spp. The majority of samples were negative for *P. ramorum* using both real-time PCR and traditional plating (91%), which is consistent with our experience of the proportion of samples submitted to CSL with *P. ramorum*-like symptoms in which *P. ramorum* is not detected. In this study, *P. ramorum* was identified only in rhododendron and viburnum samples, reflecting the fact that these were the predominant hosts in the United Kingdom at the time of the study. However, *P. ramorum* has been identified, using the DNA extraction and real-time PCR method described, in inoculated and naturally infected plants of a number of other species (including artificially inoculated *Taxus baccata* and a *Camellia* sp.; data not shown). The COX internal control assay has been used to confirm the successful extraction of PCR-amplifiable DNA, using the method described, from a range of other potential hosts sampled in this study (including *Corylus*, *Hamamelis*, *Hydrangea*, *Kalmia*, *Leucothoe*, *Peronskia*, *Phygelius*, *Picea*, *Pieris*, *Quercus*, *Sambucus*, *Syringa*, *Thuja*, and *Vaccinium* spp.). Infected material was not available for these hosts; however, because PCR-amplifiable plant DNA was extracted successfully, it is likely that pathogen DNA also could be extracted from these species using the same method.

Direct real-time PCR testing of plant material offers the potential to reduce testing times from 6 to 9 days to 1 to 2 days, as well as overcoming concerns about isolation as a reliable detection method from old, dried out, or dirty samples. Real-time PCR testing of plant material, however, does not ascertain pathogen viability, only the presence of its DNA. A move toward disease control as opposed to eradication may see the increased use of pesticides to suppress symptoms and protect plants; this will affect viability but not the presence of *P. ramorum* DNA and, therefore, direct real-time PCR testing of plant material alone may not always be appropriate. However, real-time PCR has the potential to allow large numbers of samples to be screened quickly and cheaply, especially if the majority of samples are likely to be negative. In the United Kingdom, <25% of samples submitted for *P. ramorum* testing are positive for *P. ramorum* confirmatory testing by isolation of real-time PCR positives could be used as a viability check if required for these, thus significantly reducing overall costs and time to deliver re-

sults. The method described here should prove to be extremely useful in survey work, in identification of *P. ramorum* in single samples, and in investigating many aspects relating to the biology of *P. ramorum*.

ACKNOWLEDGMENTS

Funding for this work was provided by Plant Health Division of Defra and work was performed under license number PHL 251A/4736 (03/2004). We thank all those who supplied isolates (Table 1), as well as V. Barton, P. Beales, I. Barker, G. Budge, E. Hobden, P. Giltrap, L. Walker, and K. Walsh at CSL for their invaluable laboratory and editorial support.

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