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Development of a real-time PCR assay for detection of *Phytophthora kernoviae* and comparison of this method with a conventional culturing technique

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Abstract *Phytophthora kernoviae* is a recently described pathogen causing leaf blight, aerial dieback and bleeding cankers on trees and shrubs in parts of Great Britain and Ireland and recently reported in New Zealand. This paper describes the development of a TaqMan real-time PCR assay based on internal transcribed spacer (ITS) sequence to aid diagnosis of this pathogen in culture and in plant material. The assay showed no cross reaction with 29 other *Phytophthora* species, including the closely related species *P. boehmeriae*, and detected at least 1.2 pg of *P. kernoviae* DNA per reaction. A rapid and simple method can be used to extract DNA prior to testing by real-time PCR, and a plant internal control assay can be used to aid interpretation of negative results. A comparison of real-time PCR and plating for 526 plant samples collected in the UK indicated that this assay is suitable for use in routine screening for *P. kernoviae*.

Keywords Diagnostic sensitivity · Diagnostic specificity · Plant health · Rhododendron

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Introduction

Phytophthora kernoviae is a recently described pathogen causing aerial dieback of a range of woodland, heathland and ornamental plants, and extensive bleeding stem lesions on trees in parts of Great Britain (Brasier et al. 2005; Beales et al. 2006). The known host range of *P. kernoviae* currently includes over 30 species (Widmer 2010). The pathogen was first discovered in historic woodland gardens in Cornwall in autumn 2003 as part of the Department for Environment, Food and Rural Affairs (Defra) and the Forestry Commission's campaign to eradicate another recently introduced pathogen, *P. ramorum*, the causal agent of sudden oak death. Emergency legislation was enacted in the UK to prevent its introduction to disease-free areas and to control it in infested areas (Anonymous 2004). This requires intensive inspection of known susceptible hosts that include European beech (*Fagus sylvatica*), common woodland understorey plants such as *Rhododendron ponticum* (Brasier et al. 2005) and a range of ornamental shrubs and trees including *Gevuina avellana, Liriodendron tulipifera, Magnolia stellata, Michelia doltsopa, Pieris formosa*, *Drimys winteri*, *Quercus ilex* and *Q. robur* (Beales et al. 2006), in addition to other susceptible hosts. Following identification of *P. kernoviae,* statutory plant health action is taken whenever the pathogen is found. Measures include destruction of infected material, holding of

potentially infected stock, hygiene controls and inspection of related stocks. Since the discovery of *P. kernoviae* in 2003, there have been further findings in England mainly involving dieback of *R. ponticum* in woodlands, with a few occurrences on mature beech trees. At times the damage has been extensive and greater than that caused by *P. ramorum*. Infection of *Vaccinium myrtillus* on open heathland in Cornwall has also recently been reported (Beales et al. 2009). There have only been a limited number of outbreaks on commercial plant raising and trading premises in England, affecting a range of common rhododendron cultivars. In 2006, *P. kernoviae* was reported in New Zealand, and it has been suggested that this pathogen may have been in New Zealand since the 1950s (Ramsfield et al. 2007).

As part of international phytosanitary controls, plant material originating from sites known to be infected with *P. kernoviae* or displaying suspicious symptoms must be tested to determine whether *P. kernoviae* is the causal agent, as opposed to other species of *Phytophthora* which do not warrant statutory phytosanitary action, or other diseases or disorders that can cause similar symptoms. In England and Wales, samples collected by the Plant Health and Seeds Inspectorate (PHSI) are submitted to the Food and Environment Research Agency (Fera) laboratory in York to be tested for *P. kernoviae* and *P. ramorum*. In general, plants in England and Wales with suspicious symptoms are first screened at the point of inspection by lateral flow device (LFD) for *Phytophthora* (Lane et al. 2007). Between 2002 and 2008, over 30,000 samples were sent to Fera to be tested for *P. kernoviae* and/or *P. ramorum*.

P. kernoviae can be distinguished from other *Phytophthora* species based on morphological features when isolated onto agar (Brasier et al. 2005). However, morphological identification takes at least 1 week and requires a great deal of specialist knowledge. Detection based solely on isolation in culture is not always possible, particularly if the host material is decayed or dried out (Hayden et al. 2004, 2006; Vettraino et al. 2010). Nucleic acid-based methods, such as real-time PCR, can reduce diagnosis time to a few hours, and can enable detection in samples from which the pathogen is not culturable (Mumford et al. 2006). Such methods also have the advantage that they can be automated for high throughput testing, or adapted for use in the field (Tomlinson et al. 2005, 2007; Hughes et al. 2006a). Nucleic acid-based assays are also compatible with parallel or multiplex testing for other species that occur on the same host, such as *P. ramorum* (Hughes et al. 2006b; Schena et al. 2006). Once isolated in a pure culture, sequencing can be used to confirm the identity of *P. kernoviae* on new hosts (Beales et al. 2006, 2009).

This paper describes the development of a one-step real-time PCR (TaqMan) assay based on internal transcribed spacer (ITS) sequence for the detection of *P. kernoviae* and the results of a comparison of this method with culturing on semi-selective media for 526 plant samples collected in the field.

Materials and methods

Maintenance of isolates for testing specificity and sensitivity

Phytophthora isolates from a diverse range of species (48 isolates from 30 species) were taken from Fera culture collection or obtained from other collections. Of the species tested, *P. boehmeriae* belongs to the same clade as *P. kernoviae* (Clade 10) in the multilocus phylogeny of Blair et al. (2008). Isolates were maintained until required on carrot piece agar (CPA) (Anonymous 2006) at approximately 20°C (12 h light/12 h dark), or in water at 4°C.

Extraction and sequencing of DNA from *Phytophthora* cultures

DNA was extracted directly from CPA plates, or from cultures grown in potato dextrose broth (Oxoid, Basingstoke, UK) on an orbital shaker at approx. 100 rpm (20°C, 12 h light/12 h dark) for 2 weeks. Plugs of approx. 1 cm^2 were taken from CPA plates and DNA was extracted using the method described by Hughes et al. (2006b). Briefly, the agar plugs were subjected to shaking for 3 min in a MiniBeadbeater 8 (BioSpec Products, Bartlesville, OK, USA) with 0.5 mm diameter glass beads, followed by DNA extraction using the NucleoSpin Plant kit (Machery Nagel, Düren, Germany) following the manufacturer's protocol for fungi. For cultures grown in potato dextrose broth, mycelial mats were removed, washed twice in molecular grade sterile distilled water

then blotted dry between two sheets of sterile filter paper. Mycelium (approximately 0.2 g) from each mat was placed in an extraction bag (Bioreba, Reinach, Switzerland) with 8–10 volumes of C1 buffer from the NucleoSpin Plant kit and ground using a HOMEX 6 homogeniser (Bioreba). DNA was extracted from 300 µl of the homogenised material using the NucleoSpin Plant kit as above. Extracts for sequencing or testing assay sensitivity were quantified using an Ultrospec 2000 spectrophotometer (Pharmacia, Peapack, NJ, USA), and extracts for sequencing were adjusted to 100 ng μl^{-1}. The internal transcribed spacer (ITS 1 and 2) and 5.8S regions for the nrRNA gene were sequenced for the 18 isolates indicated in Table 1 according to Hughes et al. (2000). In addition, previously published sequences listed in Table 1 were obtained from GenBank; in total, sequences representing 15 species were included in the alignment used for primer and probe design.

TaqMan primer and probe design

The sequences listed in Table 1 were aligned using the Clustal V method of the MegAlign program (DNASTar, Madison, WI, USA). Primers and a TaqMan probe for *P. kernoviae* were designed using Primer Express v 2.0 software (Applied Biosystems, Foster City, CA, USA) within the region of the ITS sequence identified as being most divergent between the species in the alignment. Primer and probe sequences are shown in Table 2. In addition to the *P. kernoviae*-specific assay, two control assays were also used: an assay designed within the conserved 5.8S region of the nrRNA gene of *Phytophthora* spp. and other oomycetes (including *Pythium* spp.), and an assay designed in a conserved region of the plant cytochrome oxidase (COX) gene (Hughes et al. 2006b).

TaqMan real-time PCR

All real-time PCR reactions were carried out in 96-well plates on an ABI 7900HT (Applied Biosystems) using TaqMan Core Reagents (Applied Biosystems). Real-time PCR reactions using the newly designed *P. kernoviae* assay, the 5.8S assay or the COX assay consisted of 1 x Buffer A and 0.025 U μl^{-1} AmpliTaq Gold, plus 0.2 mM each dNTP, 5.5 mM $MgCl_2$, 300 nM forward primer, 300 nM reverse primer, and 100 nM probe. For each reaction, 1 µl of DNA extract was added, giving a final volume of 25 µl. Cycling conditions were 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Real-time PCR reactions using the *P. kernoviae* assay of Schena et al. (2006) targeting the ras-related protein (*Ypt*1) gene consisted of 1 x Buffer A and 0.032 U μl^{-1} AmpliTaq Gold, plus 0.2 mM each dNTP, 5 mM

Table 1 Sequences used in internal transcribed spacer (ITS) sequence alignment for primer design

Phytophthora species	GenBank accession number/Fera reference
P. boehmeriae	AF228076
P. cambivora	AF266763, AJ007040, AF139396, AF087479
P. cinnamomi	AF087478, AF266764
P. cactorum	L41357, AF087480, AF228077, AF266772
P. citricola	AY769942[a], AY769941[a], AY785953[a], AY785954[a], AJ007370, AF266788, L41375,
P. cryptogea	AF228090, AF087476, AF228099, AF228100, AF228101, AF087477
P. heveae	AF266770
P. idaei	CC 1366[a]
P. ilicis	AY423297
P. kernoviae	DQ002008[a], DQ002009[a], DQ00202010[a], DQ002011[a], DQ066920[a], DQ066921[a], AY940661
P. lateralis	AF266804, AF287256, AY785952[a]
P. nemorosa	AY332654
P. nicotianae	AF228086, AF228085, AF264776, AF228085
P. pseudosyringae	AY366462, AY366463, AY230190, AY242980
P. ramorum	AY785958[a], AY785959[a], AY785960[a], AY785955[a], AY785956[a], AY785957[a]

[a] Sequences generated for the purposes of this work

Table 2 Sequences of primers and TaqMan probes

Primer/probe	Sequence (5′-3′)
Pkern 615 F	CCGAACAATCTGCTTATTGTGGCT
Pkern 722R	GTTCAAAAGCCAAGCTACACACTA
Pkern 606 T probe[a]	TGCTTTGGCGTTTGCGAAGTTGGT
5.8S F[b]	TGTCTAGGCTCGCACATCGA
5.8S R[b]	GATGACTCACTGAATCCTGCAATT
5.8S probe[b, c]	ACGCTGCGAACTGCGATACGTAATGC
COX F[b]	CGTCGCATTCCAGATTATCCA
COX RW[b]	CAACTACGGATATATAAGRRCCRRAACTG
COX probe[b, d]	AGGGCATTCCATCCAGCGTAAGCA
Yptc3F[e]	GCTCCAAATTGTACGTCTCCG
Yptc4R[e]	AACCAATTAGTCACGTGCTGATATAAA
YptcP probe[e, f]	ATCATAGCCCTTCCCAGAAGCTGTCACA

[a] FAM reporter (5′); TAMRA quencher (3′)

[b] From Hughes et al. (2006b)

[c] JOE reporter (5′); BHQ1 quencher (3′)

[d] VIC reporter (5′); TAMRA quencher (3′)

[e] From Schena et al. (2006)

[f] Yakima Yellow reporter (5′); BHQ1 quencher (3′)

$MgCl_2$, 330 nM forward primer, 330 nM reverse primer, and 130 nM probe. For this assay only, cycling conditions were 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 62.5°C (Schena et al. 2006). Negative controls containing water instead of DNA were included in all runs. A default ΔRn threshold setting of 0.2 fluorescence units was used to interpret results in terms of cycle threshold (Ct) values (the cycle at which fluorescence exceeds the threshold value).

All reactions were carried out with at least two replicates in each run. Experiments to characterise assay specificity and limit of detection were repeated at least once. DNA extracts from 574 field samples were each tested for *P. kernoviae* and COX in a single real-time PCR run with duplicate reactions.

Extraction of DNA from plant material

DNA was extracted from aseptically excised samples of plant material using a KingFisher ML instrument (Thermo Scientific, Waltham, MA, USA) as described by Tomlinson et al. (2010). Briefly, plant material (typically 200–500 mg) was homogenized in 5 ml of Buffer C1 from the NucleoSpin Plant kit, incubated at 65°C for 30 min and centrifuged for 2 min at 6000 x g. To extract DNA from the clarified lysates, 1 ml Qiagen PB Binding Buffer (Qiagen, Hilden, Germany) and 75 μl Magnesil paramagnetic particles (PMPs) (Promega, Madison, WI, USA) were added and the samples were processed using a KingFisher ML to wash the PMPs three times in 70% ethanol followed by elution of the DNA in 200 μl molecular grade water.

Isolation of *Phytophthora* spp. from field samples

Samples were tested using the method described in the EPPO diagnostic protocol for *P. ramorum* (Anonymous 2006). Samples were examined for typical symptoms, and pieces of tissue (approx. 1 cm^2) were aseptically excised from the leading edges of lesions. The pieces of tissue were placed in a small plastic bag with approx. 50 ml of distilled water for at least 15 min to aid re-hydration, during which time the bags were shaken vigorously one to three times for 15–20 s. After this time the water was changed twice to remove dislodged debris. The tissue was plated out on semi-selective P_5ARP(H) agar (Jeffers and Martin 1986), and the plates were examined microscopically for growth of *P. kernoviae* after 6 days incubation (approx. 20°C, 12 h light/12 h dark) (Erwin and Ribeiro 1996; Brasier et al. 2005).

Comparison of testing by isolation and real-time PCR

Samples of plant material with suspicious symptoms were collected in the field by the PHSI and sent to Fera for testing. Samples were dispatched in sealed plastic bags containing a small piece of damp tissue. For rhododendron samples, four pieces of tissue were excised and each piece was split into two approximately equal parts: one half was plated out onto P_5ARP(H) and the remaining pieces were subjected to DNA extraction. Samples of other hosts were treated as follows: material was placed in a clean disposable plastic box two thirds filled with Petri's mineral solution (Jeffers and Martin 1986). Pieces (each

approx. 1.5 cm×2 cm) of *Rhododendron catawbiense* leaf were floated on top and incubated at room temperature for 3 days. After this time, the rhododendron leaf pieces were removed, divided into two and subjected to DNA extraction and plating out onto P_5ARP(H), as described above. The DNA extracts were tested by real-time PCR using the *P. kernoviae* ITS assay and the COX plant control assay.

Results

Assay specificity

In total, 48 isolates from 30 species of *Phytophthora* were tested using the *P. kernoviae* real-time PCR assay and the 5.8S control assay (Table 3). The results for the 5.8S assay showed that all extracts tested contained amplifiable DNA, with Ct values ranging from 15.79 to 18.69 (mean values for duplicate reactions), indicating that the amounts of DNA present in each reaction were within an approximately 10-fold range. Using the *P. kernoviae*-specific assay, *P. kernoviae* isolates gave mean Ct values between 21.19 and 22.53. The difference in Ct values observed using the 5.8S and *P. kernoviae*-specific assays are suggestive of a difference in sensitivity between these assays. No amplification was observed for any of the non-target species tested including *P. boehmeriae,* the most closely related of the species tested based on both ITS sequence and the multi-locus phylogeny of Blair et al. (2008).

Assay sensitivity

The sensitivity of the assay was assessed by testing triplicate reactions of a ten-fold dilution series of DNA extracted from a culture of *P. kernoviae* ranging from 12 ng μl^{-1} to 1.2 pg μl^{-1} (Fig. 1). The lowest amount of DNA detected was 1.2 pg. A dilution series of *P. kernoviae* DNA was also tested with the addition of 1 μl of DNA extract from healthy rhododendron (containing approx. 2 ng μl^{-1} DNA) to each reaction. No increase in Ct values or decrease in amplification efficiency were observed (data not shown) indicating that the assay was not adversely affected by the presence of plant DNA or potentially inhibitory substances (such as polyphenolics) which may be co-extracted from plant material. In a direct comparison

Table 3 TaqMan real-time PCR results for *Phytophthora* isolates tested with the *P. kernoviae*-specific assay and generic 5.8S assay

Species	Fera culture collection reference	Ct values	
		5.8S assay	*P. kernoviae* assay
P. boehmeriae	2273	16.28	–
P. boehmeriae	2157	16.56	–
P. botryosa	2201	18.04	–
P. cactorum	1254	17.93	–
P. cactorum	2151	18.09	–
P. cambivora	1221	17.06	–
P. cambivora	1693	16.60	–
P. cinnamomi	1226	16.23	–
P. cinnamomi	1710	16.03	–
P. citricola	1531	16.25	–
P. citricola	2098	16.43	–
P. citricola	2081	16.86	–
P. citricola	1530	17.07	–
P. citrophthora	1705	16.07	–
P. cryptogea	1708	16.95	–
P. erythroseptica	2202	18.02	–
P. europaea	2159	17.39	–
P. rubi	2106	16.77	–
P. gonopodyides	2196	16.28	–
P. heveae	1700	17.04	–
P. hibernalis	2198	17.45	–
P. insolita	2274	17.65	–
P. ilicis	2194	17.37	–
P. kernoviae	2306	17.15	21.19
P. kernoviae	2169	16.98	21.26
P. kernoviae	2111	17.61	22.53
P. lateralis	2199	16.96	–
P. lateralis	2200	18.69	–
P. macrochlamydospora	2191	17.10	–
P. megasperma	2158	16.26	–
P. nemorosa	2193	17.18	–
P. nicotianae	1698	17.72	–
P. palmivora	1706	17.50	–
P. parasitica	1709	16.48	–
P. pseudosyringae	2192	16.15	–
P. pseudosyringae	2369	15.79	–
P. quercina	2197	17.10	–
P. ramorum (EU origin[1])	P1376	17.22	-
P. ramorum (EU origin)	1684	17.55	–
P. ramorum (US origin[2])	P1579	17.79	–

Table 3 (continued)

Species	Fera culture collection reference	Ct values	
		5.8S assay	*P. kernoviae* assay
P. ramorum (US origin)	P1349	17.94	–
P. richardiae	2189	17.77	–
P. richardiae	2188	17.12	–
P. syringae	1699	17.84	–
P. syringae	1702	16.34	–
P. syringae	2238	16.93	–
P. syringae	2239	16.53	–
P. uliginosa	2156	17.25	–
Water control	–	–	–

Mating type for *P. ramorum* isolates confirmed by Forest Research as A1 ([1]) and A2 ([2]). Cycle threshold (Ct) values are mean values for duplicate reactions;—indicates a negative result

of the ITS assay and a previously described *Ypt* assay for *P. kernoviae* (Schena et al. 2006), the ITS assay was found to be around 1 order of magnitude more sensitive (Fig. 1), as might be predicted for an assay targeting a multi-copy region such as ITS.

Comparison of testing by isolation and real-time PCR

A total of 574 samples submitted by the PHSI from England and Wales were used to perform a comparison between isolation on semi-selective media followed by morphological identification and real-time PCR testing of DNA extracted directly from the plant material. The predominant host was rhododendron (436 samples); others included camellia (29 samples), *Larix* (23 samples), viburnum (10 samples), and 28 other genera including ornamentals, and amenity and forest trees. Non-rhododendron hosts were processed as described above. DNA extraction was successful, as indicated by a positive result for *P. kernoviae* and/or COX, for 526 out of 574 samples (91.6%).

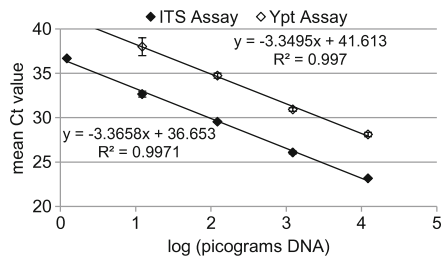

Fig. 1 TaqMan real-time PCR results showing limit of detection and efficiency of the *Phytophthora kernoviae* internal transcribed spacer (ITS) assay and the assay described by Schena et al. (2006) targeting the ras-related protein (*Ypt*1) gene. Mean Ct values are for triplicate reactions; error bars show standard deviations

Real-time PCR results for the 526 samples for which DNA extraction was successful were compared with the results of isolation (Fig. 2). Of these samples, 19 were positive for *P. kernoviae* by real-time PCR, with mean Ct values ranging between 20.37 and 37.31. Of these samples, 15 tested positive for *P. kernoviae* by plating. Ct values for the 15 samples which were positive by both real-time PCR and plating ranged from 20.37 to 35.43; Ct values for the four samples which were positive by real-time PCR but negative by plating ranged from 33.46 to 37.31. The 507 samples which were negative for *P. kernoviae* by real-time PCR had COX Ct values ranging from 17.18 to 39.42, representing a wide range of DNA concentrations and/or different levels of inhibition. Four samples were positive for *P. kernoviae* by plating but tested negative by real-time PCR, with COX Ct values ranging from 28.73 to 33.31. It is possible that these results indicate uneven distribution of the pathogen in the samples that were split for plating and DNA extraction. These results are summarised in Fig. 2a. The diagnostic sensitivity and specificity of the *P. kernoviae* TaqMan assay were found to be 78.9% and 99.2%, respectively.

In practice, a Ct value cut-off can be used to identify samples for which the real-time PCR result is inconclusive. Samples for which the *P. kernoviae* Ct value exceeds the cut-off can be subjected to plating in order to clarify the result. It is also useful to identify a cut-off for the plant control (COX) assay in order to identify samples for which DNA extraction was inefficient. Results can be obtained for these samples either by plating or by re-extraction and re-testing by real-time PCR. For example, using Ct value cut-offs of 35 and 30 for the *P. kernoviae* and COX assays, respectively, 58 samples were identified as requiring plating for clarification of the TaqMan result (in addition to the 48 samples for which DNA extraction failed). The diagnostic sensitivity and specificity of the *P. kernoviae* assay calculated for the remaining 468 samples are 93.3% and 99.8%, respectively (Fig. 2b). Due to the low prevalence of *P. kernoviae* in the UK it is possible in practice to

a

real-time PCR	plating +	plating -	Total
+	15 (A)	4 (B)	19
-	4 (C)	503 (D)	507
Total	19	507	526

b

real-time PCR	plating +	plating -	Total
+	14 (A)	1 (B)	15
-	1 (C)	452 (D)	453
Total	15	453	468

Fig. 2 Summary of the comparison of TaqMan real-time PCR and plating followed by morphological examination for detection of *Phytophthora kernoviae* in 574 samples collected during the course of routine inspection in the UK. Results were not obtained for 48 of the 574 samples due to failure of DNA extraction. +: positive result; -: negative result. (a) results interpreted without Ct value cut-offs: diagnostic sensitivity (A/(A+C))=78.9%; diagnostic specificity (D/(B+D))=99.2%. (b) results interpreted with Ct values cut-offs of 35 and 30 for the *P. kernoviae* and COX assays, respectively: diagnostic sensitivity=93.3%; diagnostic specificity=99.8%. Using these cut-off values, an additional 58 samples require plating to obtain a result

confirm all real-time PCR positives, resulting in an effective diagnostic specificity of 100%. The prevalence of *P. kernoviae* in the samples tested is particularly low because many of the samples were submitted on suspicion of the more prevalent pathogen *P. ramorum*: out of 574 samples, 28.7% were found to be positive for *P. ramorum*, compared with 3.8% for *P. kernoviae* (data not shown).

Discussion

This paper describes the development of a real-time PCR assay for detection of *P. kernoviae*. Since its discovery in 2003, and implementation of emergency EU legislation in 2004, there has been an international requirement to test susceptible hosts for this pathogen. In the UK more than 30,000 samples have been tested for *Phytophthora* since 2002, and many European countries, as well as the USA, Australia and New Zealand, have implemented monitoring for *P. kernoviae* and *P. ramorum*.

The *P. kernoviae* real-time PCR assay did not amplify 29 non-target species of *Phytophthora*, and the limit of detection was approximately 1.2 pg *P. kernoviae* DNA. This limit of detection is comparable to that of the *P. ramorum* real-time PCR assay described by Hughes et al. (2006b). Schena et al. (2006) reported higher sensitivity for a real-time PCR assay for *P. kernoviae* targeting the ras-related (*Ypt*1) gene (limit of detection approx. 100 fg); however, in a direct comparison of the two assays we observed the ITS assay to be approximately one order of magnitude more sensitive than the *Ypt* assay (Fig. 1). This discrepancy is likely to represent differences in the purity of DNA used here and in the initial description of the *Ypt* assay.

It has been reported that the ITS region is insufficiently variable to allow specific discrimination of all *Phytophthora* species using assays targeting this region alone (Schena et al. 2006). Kroon et al. (2004) and Blair et al. (2008) have used other loci for the construction of phylogenies for the genus. Other regions that have been targeted for PCR-based detection of *Phytophthora* species include mitochondrial sequences (*cox*I and II), β-tubulin and elicitin (Martin et al. 2004; Bilodeau et al. 2007), and the *Ypt*1 gene (Schena et al. 2006). However, the ITS-based assay described here was found to be suitably sensitive and specific for testing infected plant material collected at outbreak sites in the UK.

The protocol for testing plant material incorporated an internal control assay designed to detect DNA from the host plant. This helps to prevent false negatives by identifying those samples for which DNA extraction has not been effective. The extraction method used is intended for relatively high throughput extraction in a partially automated process, so may not be optimal for every sample type encountered in the course of routine testing. It was observed that DNA extraction was successful for approximately 92% of the samples processed (for approximately 8% of samples, both the *P. kernoviae* assay and the COX assay were negative). The results of the COX assay may reflect both a broad range of DNA concentrations and different levels of inhibitors in the DNA extracts tested, perhaps reflecting the diverse nature of the samples submitted for testing. DNA extracted from healthy rhododendron was not observed to have an adverse effect on the sensitivity or efficiency of the

P. kernoviae assay. However, extracts from partially necrotic samples collected in the field are likely to contain higher levels of potential inhibitors, and furthermore the COX and *P. kernoviae* assays may be differently susceptible to inhibition. Extraction of amplifiable DNA is likely to be most robust when samples are relatively clean rather than severely necrotic, so optimal collection and sampling protocols may help to maximise the efficiency of real-time PCR-based testing.

The *P. kernoviae* assay was found to have high analytical sensitivity and specificity when testing DNA extracted from pure cultures. When testing plant material collected in the field, however, a pragmatic approach can facilitate accurate and efficient routine testing of a large number of samples. A Ct value cut-off can be set above which positive results are considered to be inconclusive, in order to prevent false positive results caused by cross-contamination between samples or other user errors. Similarly, a cut-off for the plant control assay can help to avoid false negative results caused by inefficient DNA extraction for individual samples. Using this approach, the real-time PCR assay could be used to test all samples as they are received, with a proportion of samples being subjected to culturing for clarification or confirmation of the result. Using Ct value cut-offs of 35 and 30 for the *P. kernoviae* and COX assays, respectively, the real-time PCR method was found to have a diagnostic sensitivity of 93.3% and a diagnostic specificity of 99.8% (Fig. 2b) in a direct comparison with culturing for 468 samples. Out of 574 samples received, DNA extraction was unsuccessful for 48 samples (8.4%) and 58 samples gave ambiguous real-time PCR results (10.1%). Setting even more conservative Ct value cut-offs of 33 and 28 for the *P. kernoviae* and COX assays, respectively, resulted in calculated sensitivity and specificity of 100%, with ambiguous real-time PCR results for 83 samples (14.5%). Appropriate cut-offs can be selected based on requirements (statutory or otherwise) to avoid or eliminate false positive and/or negative results, as well as considerations of the speed and cost of testing by real-time PCR or culturing (assumed here to be the 'gold standard' method).

The assay described in this paper could be a useful tool in determining the geographical distribution of *P. kernoviae* and helping to prevent further spread of this highly damaging pathogen, and has been adopted for routine detection of *P. kernoviae* at Fera. High throughput screening of samples using this method can be combined with traditional culturing techniques for clarification of ambiguous results and for confirming the presence of the pathogen in new hosts or at new outbreak sites. Due to the generic nature of real-time PCR-based methods, the *P. kernoviae*-specific assay described here can be readily performed in parallel with an assay for detection of *P. ramorum* (Hughes et al. 2006b). Furthermore, these laboratory methods could be used in conjunction with on-site tests such as *Phytophthora* spp. LFD (Lane et al. 2007; Tomlinson et al. 2010) to maximise the overall efficiency and accuracy of testing.

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References

Anonymous (2004). Statutory instrument No. 3367. The Plant Health (*Phytophthora kernovii* Management Zone) (England) order 2004. HMSO. ISBN 0110513878

Anonymous. (2006). EPPO diagnostic protocol for regulated pests: *Phytophthora ramorum*. *EPPO Bulletin, 36*, 145–155.

Beales, P. A., Lane, C. R., Barton, V. C., & Giltrap, P. M. (2006). *Phytophthora kernoviae* on ornamentals in the UK. *EPPO Bulletin, 36*, 377–379.

Beales, P. A., Giltrap, P. M., Payne, A., & Ingram, N. (2009). A new threat to UK heathland from *Phytophthora kernoviae* on *Vaccinium myrtillus* in the wild. *Plant Pathology, 58*, 393.

Bilodeau, G. J., Lévesque, C. A., de Cock, A. W. A. M., Duchaine, C., Brière, S., Uribe, P., et al. (2007). Molecular detection of *Phytophthora ramorum* by real-time polymerase chain reaction using TaqMan, SYBR Green and molecular beacons. *Phytopathology, 97*, 632–642.

Blair, J. E., Coffey, M. D., Park, S. Y., Geiser, D. M., & Kang, S. (2008). A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. *Fungal Genetics and Biology, 45*, 266–277.

Brasier, C. M., Beales, P. A., Kirk, S. A., Denman, S. & Rose, J. (2005). *Phytophthora kernoviae* sp. Nov., an invasive pathogen causing bleeding stem lesions on forest trees and foliar necrosis of ornamentals in the UK. *Mycological Research, 109*, 853–859

Erwin, D. C., & Ribeiro, O. K. (1996). *Phytophthora diseases worldwide*. St. Paul, MN, USA: American Phytopathological Society.

Hayden, K. J., Rizzo, D., Tse, J., & Garbelotto, M. (2004). Detection and quantification of *Phytophthora ramorum*

from Californian forests using a real-time polymerase chain reaction assay. *Phytopathology, 94*, 1075–1083.

Hayden, K., Ivors, K., Wilkinson, C., & Garbelotto, M. (2006). TaqMan chemistry for *Phytophthora ramorum* detection and quantification, with a comparison of diagnostic methods. *Phytopathology, 96*, 846–854.

Hughes, K. J. D., Fulton, C. E., McReynolds, D., & Lane, C. R. (2000). Development of new PCR primers for identification of *Monilinia* species. *EPPO Bulletin, 30*, 507–511.

Hughes, K. J. D., Giltrap, P. M., Barton, V. C., Hobden, E., Tomlinson, J. A., & Barber, P. (2006). On-site real time PCR detection of *Phytophthora ramorum* causing dieback of *Parrotia persica* in the UK. *Plant Pathology, 55*, 813.

Hughes, K. J. D., Tomlinson, J. A., Griffin, R. L., Boonham, N., Inman, A. J., & Lane, C. R. (2006). Development of a One-Step Real-Time PCR Assay for Diagnosis of *Phytophthora ramorum*. *Phytopathology, 96*, 975–981.

Jeffers, S. N., & Martin, S. B. (1986). Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease, 70*, 1038–1043.

Kroon, L. P. N. M., Bakker, F. T., van den Bosch, G. B. M., Bonants, P. J. M., & Flier, W. G. (2004). Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology, 41*, 766–782.

Lane, C. R., Hobden, E., Walker, L., Barton, V. C., Inman, A. J., Hughes, K. J. D., Swan, H., Colyer, A. & Barker, I. (2007). Evaluation of a rapid diagnostic field test kit for identification of *Phytophthora* species, including *P. ramorum* and *P. kernoviae* at the point of inspection. *Plant Pathology, 56,* 828–835

Martin, F. N., Tooley, P. W., & Blomquist, C. (2004). Molecular detection of *Phytophthora ramorum*, the causal agent of sudden oak death in California, and two additional species commonly recovered from diseased plant material. *Phytopathology, 94*, 621–631.

Mumford, R., Boonham, N., Tomlinson, J., & Barker, I. (2006). Advances in molecular phytodiagnostics - new solutions for old problems. *European Journal of Plant Pathology, 116*, 1–19.

Ramsfield, T. D., Dick, M. A., Beever, R. E. & Horner, I. J. (2007). *Phytophthora kernoviae* – of Southern Hemisphere origin? Abstracts of the Fourth IUFRO working party meeting 7.02.09. *Phytophthoras* in Forests and Natural Ecosystems, Monterey, CA, USA August 26–31 2007.

Schena, L., Hughes, K. J. D. & Cooke, D. E. L. (2006). Detection and quantification of *Phytophthora ramorum, P. kernoviae, P. citricola,* and *P. quercina* in symptomatic leaves by multiplex PCR. *Molecular Plant Pathology, 7,* 365–379

Tomlinson, J. A., Barker, I., & Boonham, N. (2007). Faster, simpler, more-specific methods for improved detection of *Phytophthora ramorum* in the field. *Applied and Environmental Microbiology, 73*, 4040–4047.

Tomlinson, J. A., Boonham, N., Hughes, K. J. D., Griffin, R. L., & Barker, I. (2005). On-site DNA extraction and real-time PCR for detection of *Phytophthora ramorum* in the field. *Applied and Environmental Microbiology, 71*, 6702–6710.

Tomlinson, J. A., Dickinson, M., Hobden, E., Robinson, S., Giltrap, P. M. & Boonham, N. (2010). A five-minute DNA extraction method for expedited detection of *Phytophthora ramorum* following prescreening using *Phytophthora* spp. lateral flow devices. *Journal of Microbiological Methods, 81,* 116–120

Vettraino, A. M., Sukno, S., Vannini, A., & Garbelotto, M. (2010). Diagnostic sensitivity and specificity of different methods used by two laboratories for the detection of *Phytophthora ramorum* on multiple natural hosts. *Plant Pathology, 59*, 289–300.

Widmer, T. L. (2010). *Phytophthora kernoviae* oospore maturity, germination, and infection. *Fungal Biology, 114*, 661–668.