MOLECULAR PLANT PATHOLOGY (2006) **7**(5), 365–379 DOI: 10.1111/J.1364-3703.2006.00345.X

Detection and quantification of *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in symptomatic leaves by multiplex real-time PCR

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SUMMARY

New species of *Phytophthora* such as *Phytophthora ramorum*, *P. kernoviae* and *P. quercina* together with *P. citricola* are plant pathogens which impact on forest health, natural ecosystem stability and international trade. A real-time multiplex PCR approach based on TaqMan PCR was developed to simultaneously identify and detect these four *Phytophthora* species. Specific primers and probes labelled with FAM (*P. ramorum*), Yakima Yellow (*P. kernoviae*), Rox (*P. citricola*) and Cy5 (*P. quercina*) were designed in different regions of the ras-related protein (*Ypt*1) gene. A new set of Black Hole Quenchers (BHQ), which dissipate energy as heat rather than fluorescence, were utilized. The method proved to be highly specific in tests with target DNA from 72 *Phytophthora* isolates (35 species). For all pathogens, the detection limit was 100 fg of target DNA and was not improved utilizing a nested approach to provide a first round of amplification with *Phytophthora* spp.-specific primers. Cycle threshold (Ct) values were linearly correlated with the concentration of the target DNA (correlation coefficients ranged from 0.947 to 0.996) and were not affected by the presence of plant extracts, indicating the appropriateness of the method for qualitative and quantitative analyses. Two universal primers and a TaqMan probe were also developed to evaluate the quality and quantity of extracted DNA and to avoid false negatives. The reliability of the entire procedure was assessed using both artificially and naturally infected leaves of a range of plant species. The method, combined with a rapid procedure for DNA extraction, proved to be rapid, reliable, sensitive and cost effective as multiple pathogens were detected within the same plant extract by using different primer/probe combinations.

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INTRODUCTION

A number of *Phytophthora* species represent a significant threat to forest and natural ecosystems where they are frequently found in 'clusters' on the same site or sometimes even on the same tree (Jung *et al.*, 2002; Vettraino *et al.*, 2002, 2005). The threats are from several well-known species such as *P. cinnamomi*, *P. citricola*, *P. cambivora*, *P. ilicis*, *P. cactorum*, *P. cryptogea*, *P. megasperma* and *P. syringae* as well as from an increasing number of recently described taxa including *P. quercina*, *P. ramorum*, *P. psychrophila*, *P. uliginosa*, *P. europaea*, *P. pseudosyringae*, *P. inundata*, *P. nemorosa*, *P. alni* and *P. kernoviae* (Brasier *et al.*, 2003, 2004b, 2005; Hansen *et al.*, 2003; Jung *et al.*, 1999, 2002, 2003; Shearer *et al.* 2004; Vettraino *et al.*, 2005; Werres *et al.*, 2001). The discovery of so many new *Phytophthora* species in a relatively short time is in part attributable to improved monitoring and new detection methods although other factors such as increased movement of the pathogens in international plant trade are likely to be significant.

Four of the *Phytophthor*a species mentioned above, *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina*, are pathogens of increasing significance to the scientific community. *P. ramorum* is responsible for tree and shrub mortality in Californian forests (Martin *et al.*, 2004; Rizzo and Garbelotto, 2003; Werres *et al.*, 2001) and in nursery stock from many states across the USA (Hansen *et al.*, 2003, 2005; Parke *et al.*, 2004; Stokstad, 2004). Control strategies are complicated by the continued increase in the reported host range of *P. ramorum* (Henricot & Prior, 2004; Parke *et al.*, 2004; Tooley *et al.*, 2004). In Europe, *P. ramorum* has been frequently reported on rhododendron and other shrubs in nurseries and woodland gardens (Beales *et al.*, 2004; Lane *et al.*, 2003, 2004; Zerjav *et al.*, 2004) and recently has been isolated from a number of trees including different species of oak, beech, horse chestnut and sweet chestnut (Brasier *et al.*, 2004a). Owing to the potential threat to natural ecosystems, regulations have been introduced in USA, Canada

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and Europe to prevent introduction and/or further spread of *P. ramorum* (Anon., 2002).

P. kernoviae is a newly described and potentially serious pathogen of woodland environments first isolated from rhododendron in Cornwall (England) in November 2003 (Brasier *et al.*, 2005). *P. kernoviae* causes extensive leaf blight and dieback of rhododendron and occasionally large necrotic bleeding cankers on several beech trees. As of May 2005, *P. kernoviae* has been found on *Fagus sylvatica* (European beech), *Rhododendron ponticum* (and rhododendron hybrids), *Gevuina avellana* (Chilean hazelnut), *Liriodendron tulipifera* (tulip tree), *Magnolia stellata*, *Michelia doltsopa*, *Pieris* spp., *Drimys winteri* (winter's bark), *Quercus ilex* (holm oak) and *Q. robur* (English oak) (http://www.defra.gov.uk/planth/pestnote/kern.pdf). *P. kernoviae* has, to date, only been found in the UK and New Zealand and has been included in the alert list of the European and Mediterranean Plant Protection Organization (EPPO) as it may present a phytosanitary risk for the EPPO region (http://www.eppo.org/).

P. citricola is a commonly isolated pathogen in central Europe with a broad range of economically important hosts such as *Q. robur* and *Q. petraea* (Jung *et al.*, 2000; Thomas *et al.*, 2002) and *F. sylvatica* (Jung *et al.*, 2005; Nechwatal and Oβwald, 2001; Wang *et al.*, 2003; Werres, 1995). *P. citricola* was the most frequently isolated *Phytophthora* species on beech trees in Europe (Jung *et al.*, 2005; Nechwatal and Oβwald, 2001) and during extensive surveys to monitor *P. ramorum* in England and Wales was also the most frequently isolated *Phytophthora* species from rhododendron leaves (Hughes *et al.*, 2006).

P. quercina, first isolated in 1995 (Jung *et al.*, 1996) and described as a new species in 1999 (Jung *et al.*, 1999) is an aggressive pathogen specific to *Quercus* spp. (Jönsson *et al.*, 2003; Jung *et al.*, 1996, 2002). The pathogen is tolerant of diverse site conditions and has been reported from oaks across large parts of Europe (Balci and Halmschlager, 2003b; Cooke *et al.*, 1999, 2005; Jung *et al.*, 1999, 2000; Vettraino *et al.*, 2002) and a limited area of Asia Minor (Balci and Halmschlager, 2002, 2003a).

Considering the threat related to the dissemination of new *Phytophthora* species such as *P. ramorum*, *P. kernoviae* and *P. quercina* and the importance of a widely distributed species such as *P. citricola*, there is a clear need for reliable, accurate and rapid diagnostic methods. In recent years, conventional and real-time PCR has emerged as a major tool for the diagnosis and study of phytopathogenic fungi and has contributed to the alleviation of some of the problems associated with the detection, control and containment of plant pathogens (Schena *et al.*, 2004a,b). Molecular procedures for the identification and detection of *P. ramorum* have been developed and are in use in various laboratories in the US and Europe. Proposed methods include classical PCR methods based on the internal transcribed spacer (ITS) regions of ribosomal DNA (Hayden *et al.*, 2004) and mitochondrial gene regions (Martin *et al.*, 2004), PCR-SSCP analysis (Kong *et al.*, 2004), and PCR-RFLP analysis (Martin and Tooley, 2004). Furthermore, ITS-based real-time PCR assays based on SYBR Green (Hayden *et al.*, 2004) and on TaqMan (Hughes *et al.*, 2006; Tomlinson *et al.*, 2005) have recently been described. A set of primers based on the ITS regions has been developed for the classical gel-based PCR detection of *P. quercina*, *P. citricola* and *P. cambivora* in seedlings of pedunculate oak (*Q. robur*) and European beech (*F. sylvatica*), which were artificially infected under controlled conditions (Schubert *et al.*, 1999). Similarly, a combination of baiting and PCR techniques has been utilized for the detection of *P. quercina* and *P. citricola* in soil samples from oak stands (Nechwatal *et al.*, 2001). Finally, specific TaqMan primers and probes have been recently designed for *P. kernoviae*, and their evaluation on pure cultures and plant samples is in progress (K.J.D. Hughes *et al.*, personal communication). All mentioned diagnostic systems are aimed at the detection of single pathogens; however, a method capable of the simultaneous detection of more than one species offers further advantages (Lievens *et al.*, 2003), in particular for testing programmes dealing with thousands of samples from national nursery surveys when high throughput and reliability are critical, which simultaneous detection (multiplex PCR assay) can offer.

In conventional multiplex PCR, discrimination of amplified fragments relies on obtaining products of different sizes that can be distinguished by gel electrophoresis. However, different size products amplify with different efficiencies. By contrast, with real-time specific detection methods, differentiation can be achieved using different fluorescent dyes, and therefore amplicons of the same length can be used. Real-time PCR chemistries utilized to detect and study phytopathogenic micro-organisms can be grouped into amplicon sequence-non-specific (SYBR Green) and sequence-specific methods (TaqMan, Molecular Beacons, Scorpion PCR, etc.) (Mackay *et al.*, 2002). SYBR green is a non-specific dye that fluoresces when intercalated into double-stranded DNA, whereas amplicon sequence-specific methods are based on the labelling of primers or probes with fluorogenic molecules that allow detection of a specific amplified target fragment (Thelwell *et al.*, 2000). In both approaches, amplicons can be measured at an early stage of the reaction when the rate of amplification is still in its linear phase, enabling the quantification of the initial amount of target DNA (quantitative analyses). In plant pathology, there are only few examples of multiplex PCR applications and usually not more than two different targets are simultaneously detected (Hughes *et al.*, 2006; Ippolito *et al.*, 2004; Tooley *et al.*, 2006; Winton *et al.*, 2002). This is partially due to the difficulties related to the development of a multiplex quantitative PCR assay and to the frequent reduction in sensitivity achieved in multiplex PCR compared with separate reactions. Real-time PCR based on specific methods combines the sensitivity of PCR with the specificity of nucleic acid hybridization, making

Southern blots or sequencing to confirm identity of the amplicons unnecessary. Therefore, these recent techniques eliminate the need for post-amplification processing steps, are faster, more easily automated and avoid the use of toxic ethidium bromide. Other significant advantages of real-time PCR are the reduced potential for cross-contamination of specimens and the higher sensitivity of the reactions (Mumford *et al.*, 2000).

Among target genes proposed to identify and detect *Phytophthora* species, the ITS regions of the nuclear-encoded ribosomal RNA genes (rDNA) are the most widely used (Cooke *et al.*, 2000). However, in some cases the ITS sequences are not sufficiently variable, making the design of primers to identify and detect closely related taxa very difficult or impossible (Brasier *et al.*, 2004; Kroon *et al.*, 2004; Martin and Tooley, 2003a,b). The elicitin gene *parA*1 and the putative storage protein genes (*Lpv*) proved to be effective targets for specific detection of *P. cinnamomi* and *P. nicotianae*, respectively, but neither gene seemed to be variable enough to distinguish a broad range of species (Kong *et al.*, 2003a,b). Intergenic regions of the mitochondrial DNA (mtDNA-IGS) showed the presence of intra- and interspecific variation (Schena and Cooke, 2006; Wattier *et al.*, 2003) and one of these regions was suited to the development of specific detection methods for *P. ramorum*, *P. nemorosa* and *P. pseudosyringae* (Martin *et al.*, 2004). A limit to the use of the mtDNA-IGS regions is that they are usually very AT/GC rich, making the design of effective primers quite difficult (Schena and Cooke, 2006). Another promising target gene for the design of *Phytophthora*-specific detection methods is the ras-related protein (*Ypt*1) gene (Chen and Roxby, 1996). The non-coding regions of the *Ypt*1 gene showed sufficient variation to enable the development of molecular markers for almost all *Phytophthora* species but did not contain intraspecific variability (Schena and Cooke, 2006).

The aim of the present study was the development of a multiplex real-time PCR method for the simultaneous detection and quantification of four target pathogens (*P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina*) in leaf materials. To the best of our knowledge, this is the first time that such a complex multiplex approach has been applied in plant pathology. The simultaneous detection of *P. ramorum* and *P. kernoviae* represents an important achievement and although *P. citricola* and *P. quercina* are less commonly isolated from leaves their detection as part of the multiplex assay described here offers diagnosticians a useful tool to screen for multiple pathogens.

RESULTS

Design and assessment of primers and probes with different levels of specificity

Twenty-seven new sequences of a region of the *Ypt*1 gene containing introns 3, 4 and 5 were obtained during this study and aligned with sequences of the same gene reported by Schena and Cooke (2006) to design specific primers (Fig. 1). Alignment of sequences from a total of 70 different isolates representative of 35 different *Phytophthora* species (Table 1) showed the presence of high interspecific variability useful for the differentiation of closely related species and, with the single exception of *P. ramorum*, the absence of any intraspecific variability. In *P. ramorum* a simple sequence repeat (SSR) of cytosine (C) containing 10 C in the two American isolates and 13 C in the two European isolates analysed was found in intron 4. This polymorphic region of the gene was not utilized to develop *P. ramorum*-specific primers.

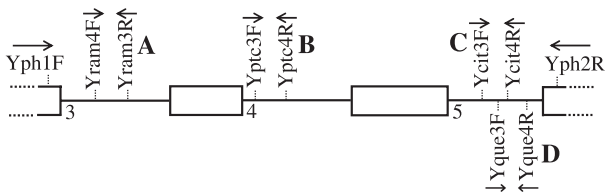

Fig. 1 Schematic representation of a region of the ras-related protein (*Ypt*1) gene containing introns 3, 4 and 5 with location of selected primers utilized in multiplex real-time PCR to detect *Phytophthora ramorum* (A), *P. kernoviae* (B), *P. citricola* (C) and *P. quercina* (D). Primers Yph1F and Yph2R were designed to amplify DNA from all *Phytophthora* species and utilized in a nested approach providing a first common amplification with these primers and a second amplification by real-time multiplex PCR. Arrows indicate the orientation of primers.

Specific primers and probes to identify *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* were designed in different regions of the *Ypt*1 gene and labelled with four different fluorophores and three different Black Hole Quenchers (BHQ) to allow the simultaneous detection of the target pathogens (multiplex real-time PCR) (Table 2 and Fig. 1). Furthermore, in an attempt to increase sensitivity of the reactions, two primers (Yph1F–Yph2R) amplifying DNA from all *Phytophthora* species (Table 1) were designed to enable the development of a nested PCR with a first common amplification with primers Yph1F–Yph2R and a second amplification by multiplex real-time PCR (Table 2 and Fig. 1). Moreover, two universal primers and a universal probe were designed in a conserved region of the 18S region of the rDNA to amplify DNA from a wide range of organisms including *Streptophyta* and *Stramenopiles*. This universal detection method was utilized to evaluate the quality of DNA extracted from environmental samples.

Specificity of all primers and probes was preliminarily assessed by means of Basic Local Alignment Search Tool (BLAST) analyses to explore all of the available DNA sequence data in international databases. Further specificity tests were conducted using 72 isolates of *Phytophthora* belonging to 35 species (Table 1), nine representative isolates of *Pythium* (*Py. pyrilobum* IMI 308312, *Py. catenulatum* IMI 323121, *Py. torulosum* IMI 308268, *Py. intermedium* Py4, *Py. dissotocum* IMI 329003, *Py. aphanidermatum* Py7, *Py. ultimum* Py8, *Py. undulatum* IMI 337230 and *Py. splendens*

Table 1 Isolates of *Phytophthora* included in the study, their designations and origins and GenBank accession numbers for sequences of a fragment of the ras-related gene (*Ypt*1).

Phytophthora species	Isolate numbers	Origin			Sequences (*Ypt*1)
		Host	Country	Year	
P. alni subsp. *alni*	SCRP2	*Alnus* sp.	UK	1995	DQ162953
	SCRP4	*Alnus* sp.	Germany	1995	DQ270297*
	SCRP8	*Alnus* sp.	France	1996	DQ270299*
P. alni subsp. *multiformis*	SCRP3	*Alnus* sp.	Netherlands	1995	DQ270307*
P. alni subsp. *uniformis*	SCRP10	*Alnus* sp.	Sweden	1996	DQ270301*
	SCRP12	*Alnus* sp.	Sweden	1996	DQ270300*
P. boehmeriae	SCRP23	*Gossypium hirsutum*	China	1998	DQ270324*
P. cactorum	IMI 296524; SCRP27	*Rubus idaeus*	Wales	1985	DQ162960
	SCRP30	*Fragaria* × *ananassa*	Sweden		DQ270309*
	SCRP35	*Fragaria* × *ananassa*	Scotland	1993	DQ270308*
	SCRP39	*Fragaria* × *ananassa*	Scotland	1988	DQ270311*
	SCRP48	*Ribes* sp.	England	1983	DQ270310*
P. cambivora	IMI 296831; SCRP67	*Rubus idaeus*	Scotland	1985	DQ162954
	SCRP75	*Fagus* sp.	UK	1995	
	SCRP80	*Castanea sativa*	Italy		DQ162955
	SCRP82	Eucalypt	Australia		DQ162956
P. capsici	IMI 352321; SCRP103	*Piper nigrum*	India	1989	DQ162972
P. cinnamomi	CBS270.55; SCRP115	*Chamaecyparis lawsoniana*	Netherlands	1993	DQ162959
	CBS342.72; SCRP118	*Persea gratissima*	California	1972	DQ270317*
	SCRP121		Australia		DQ270316*
P. citricola	SCRP130	*Rubus idaeus*	Scotland	1986	DQ162968
	SCRP136	Soil	UK	1995	DQ162969
	SCRP140	*Taxus* sp.	UK	1995	DQ162970
	SCRP143	*Quercus robur*	Germany		DQ162971
P. citrophthora	IMI 332632; SCRP179	*Actinidia chinensis*	Chile	1989	DQ162973
P. cryptogea	IMI 045168; SCRP207	*Lycopersicon esculentum*	New Zealand	1951	DQ162987
P. drechsleri	ATCC46724; SCRP232	*Beta vulgaris*	USA	1935	DQ162989
P. erythroseptica	SCRP240	*Solanum tuberosum*	Netherlands		DQ162988
P. europaea	SCRP622	*Quercus robur*	Switzerland		DQ162952
P. fragariae var. *fragariae*	SCRP245	*Fragaria* × *ananassa*	England	1945	DQ162950
	SCRP779	*Fragaria* × *ananassa*	Scotland	1979	DQ270306*
P. fragariae var. *rubi*	SCRP249	*Rubus idaeus*	Germany	1985	DQ270305*
	SCRP278	*Rubus idaeus*	USA	1987	DQ270304*
	SCRP310	*Rubus idaeus*	Sweden	1995	DQ270303*
	IMI355974; SCRP333	*Rubus idaeus*	Scotland	1985	DQ162951
	SCRP339	*Rubus idaeus*	France	1985	DQ270302*
P. idaei	CBS968.95; SCRP370	*Rubus idaeus*	Scotland	1985	DQ270312*
	IMI313727; SCRP371		England	1986	DQ270313*
	SCRP373		England	1987	DQ270314*
	SCRP376		England	1993	DQ270315*
P. ilicis	SCRP377	*Ilex aquilifolium*	UK	1995	DQ162962
	SCRP379	*Ilex aquilifolium*	UK		DQ162963
P. infestans	SC03.26.3.3	*Solanum tuberosum*	Scotland	2003	DQ162961
P. insolita	IMI288805; SCRP385	Soil	Taiwan	1979	DQ162974
P. inundata	IMI389751; SCRP644	*Salix* sp.	UK	1972	DQ162982
	IMI389750; SCRP643	*Aesculus hippocastanum*	UK	1970	DQ162983
	SCRP647	*Vitis* sp.	South America	1997	DQ162984
	SCRP649	*Alnus glutinosus*	Denmark	1995	DQ162985
P. katsurae	SCRP388				DQ162980

Table 1 *continued.*

Phytophthora species	Isolate numbers	Origin			Sequences (*Ypt*1)
		Host	Country	Year	
P. kernoviae	SCRP722	*Fagus sylvatica*	England	2003	DQ162975
	SCRP957	*Fagus sylvaticus*	England	2005	DQ270322*
	SCRP958	*Fagus sylvaticus*	England	2005	DQ270321*
	KER-CSL		England		DQ270323*
P. lateralis	IMI 040503; SCRP390	*Chamaecyparis lawsoniana*	USA	1942	DQ162991
P. medicaginis	SCRP407	*Medicago* sp.	Iran	1989	DQ162990
P. megasperma	IMI 133317; SCRP435	*Malus sylvestris*	Australia	1968	DQ162986
P. nemorosa	SCRP910			2004	DQ162965
P. nicotianae	IMI 268688; SCRP468	*Citrus* sp.	Trinidad		DQ162981
P. palmivora	SCRP526	*Hevea brasiliensis*	Thailand	1995	
P. pistaciae	IMI386658; SCRP533	*Pistacia vera*	Iran	1986	DQ162957
P. pseudosyringae	IMI390500; SCRP674	*Malus pumila*	Italy	2001	DQ162966
	SCRP734	*Fagus sylvatica*	Italy	2003	DQ162967
P. psychrophila	SCRP630	*Quercus ilex*	France		DQ162964
P. quercina	SCRP541	*Quercus robur*	Germany		DQ162976
	SCRP547	*Quercus cerris*	Germany		DQ162977
	SCRP549	*Quercus ilex*	Italy		DQ162978
	SCRP550	*Quercus robur*	Germany		DQ162979
P. ramorum	SCRP911	*Rhododendron* sp.	Scotland	2004	DQ162992
	SCRP954	*Viburnum tinus*	England	2005	DQ270319*
	SCRP955	*Lithocarpus densiflorus*	Oregon		DQ270320*
	SCRP956	*Quercus agrifolia*	USA		DQ270318*
P. sojae	SCRP555	*Glycine max*	USA	1995	DQ162958

*Accession obtained in the present study; all other sequences were reported in Schena and Cooke (2006).

IMI 391319), and DNA extracted from leaves of five different species (*R. ponticum*, *Q. ilex*, *F. sylvatica*, *Prunus avium* and *Betula pendula*). *Phytophthora* and *Pythium* species representative of the breadth of diversity in each genus were selected (Cooke *et al.*, 2000; Kroon *et al.*, 2004; Lévesque and De Cock, 2004). All primers and probes showed the desired level of specificity. Using amplification mixtures containing all specific primers and probes a precise increase in fluorescence at the expected wavelength was obtained for all the isolates of each of the four target pathogens (Fig. 2). Fluorescence remained below the threshold values for the water controls, the non-target *Phytophthora* species (Fig. 2), the nine *Pythium* species and all DNA samples extracted from uninfected leaves (data not shown). The *Phytophthora*-specific primers amplified a fragment of the expected size (approximately 470 bp) from all the species of *Phytophthora* but did not amplify from *Pythium* or plant DNA. Finally, the universal primers and probe amplified all target sequences tested.

Sensitivity of multiplex real-time PCR reactions

Sensitivity of multiplex real-time PCR reactions and standard curves for quantitative analyses were constructed, amplifying DNA from the four target pathogens (*P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina*) in a single tube. Total DNA from *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* was mixed together and serially diluted with sterile water to yield final concentrations ranging from 1 ng/μL to 1 fg/μL of DNA of each pathogen. In negative control reactions water replaced template DNA. The detection limit of real-time multiplex PCR was 100 fg of target DNA for all pathogens (Fig. 3). Standard curves showed a linear correlation between input DNA and cycle threshold (Ct) values with correlation coefficients (r^2) of 0.996 (*P. ramorum*), 0.991 (*P. kernoviae*), 0.960 (*P. citricola*) and 0.947 (*P. quercina*) (Fig. 3).

Additional experiments testing amplification of each of the four target DNAs in non-multiplex were performed, i.e. in four separate reactions with only the single set of specific primers and probe for one species. Levels of sensitivity and correlation coefficients assessed in separate reactions were as in the multiplex PCR (Fig. 4). Serially diluted DNA samples were also amplified by nested PCR combining a first amplification with the *Phytophthora*-specific primers Yph1F–Yph2R and a second amplification in which 1 μL of amplified product from the first round was utilized as template in multiplex real-time PCR. Surprisingly, the overall level of sensitivity obtained with nested PCR was

Table 2 Primers and probes used in this study.

Target species	Primers (5′–3′)	Probes (5′–3′)	Amplified fragment (bp)
Phytophthora spp.	Yph1F CGACCATKGGTGTGGACTTT Yph2R ACGTTCTCMCAGGCGTATCT		≅ 450
P. ramorum	Yram4F TTTGTCAGTGACCTCTCTCTCTCTC Yram3R GCATAAGTATAAGTCAGCAAGCCTGT	YramP AGAACACGATCCCCTCGTCAGCAGTC	87
P. kernoviae	Yptc3F GCTCCAAATTGTACGTCTCCG Yptc4R AACCAATTAGTCACGTGCTGATATAAA	YptcP ATCATAGCCCTTCCCAGAAGCTGTCACA	78
P. citricola	Ycit3F TTCGTGATACAGTACTTCAGGATTTCTG Ycit4R AACAGTGGCGCAACGGTTAG	YcitP TCGTTACCGTGGCACCCAATGCA	73
P. quercina	Yque3F GGTGCGTCTGAATTTAGATTTTCTTT Yque4R CGTATCTGCAAATCACGTGCAT	YqueP TCAGTTTGCTCCTTTCCGTGGGTCTTCT	81
Universal Probe	LS-F GGATCCATTGGAGGGCAAGT LS-R CTTAAATATACGCTATTGGAGCTGGAA	LS-P TACCGCGGCTGCTGGCACCA	69

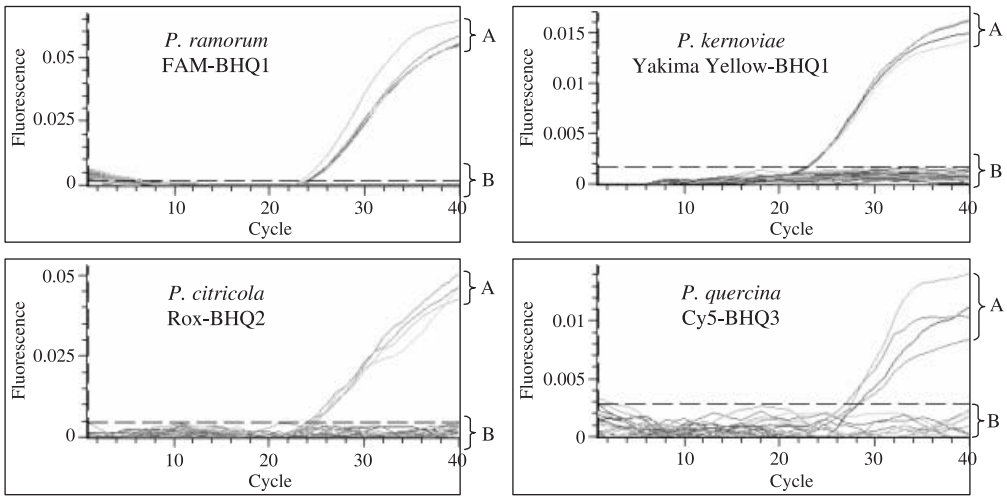

Fig. 2 Results of tests conducted to assess specificity of the multiplex real-time PCR using DNA from 72 isolates representative of 35 *Phytophthora* species (Table 1) and nine representative species of *Pythium*. A = target pathogen; B = other *Phytophthora* and *Pythium* isolates.

generally not increased over that in single real-time multiplex PCR (100 fg). In nested PCR, the increase in fluorescence occurred earlier until the concentration reached 100 fg of DNA (average Ct 19.5) but then no increase in fluorescence or extra sensitivity was obtained for any further diluted sample. The same result was confirmed in three separate experiments using different dilution series. Based on these results, the nested approach was not utilized to analyse naturally and artificially infected leaves.

To determine whether the presence of plant DNA and coextracted compounds affected the amplification of target DNA,

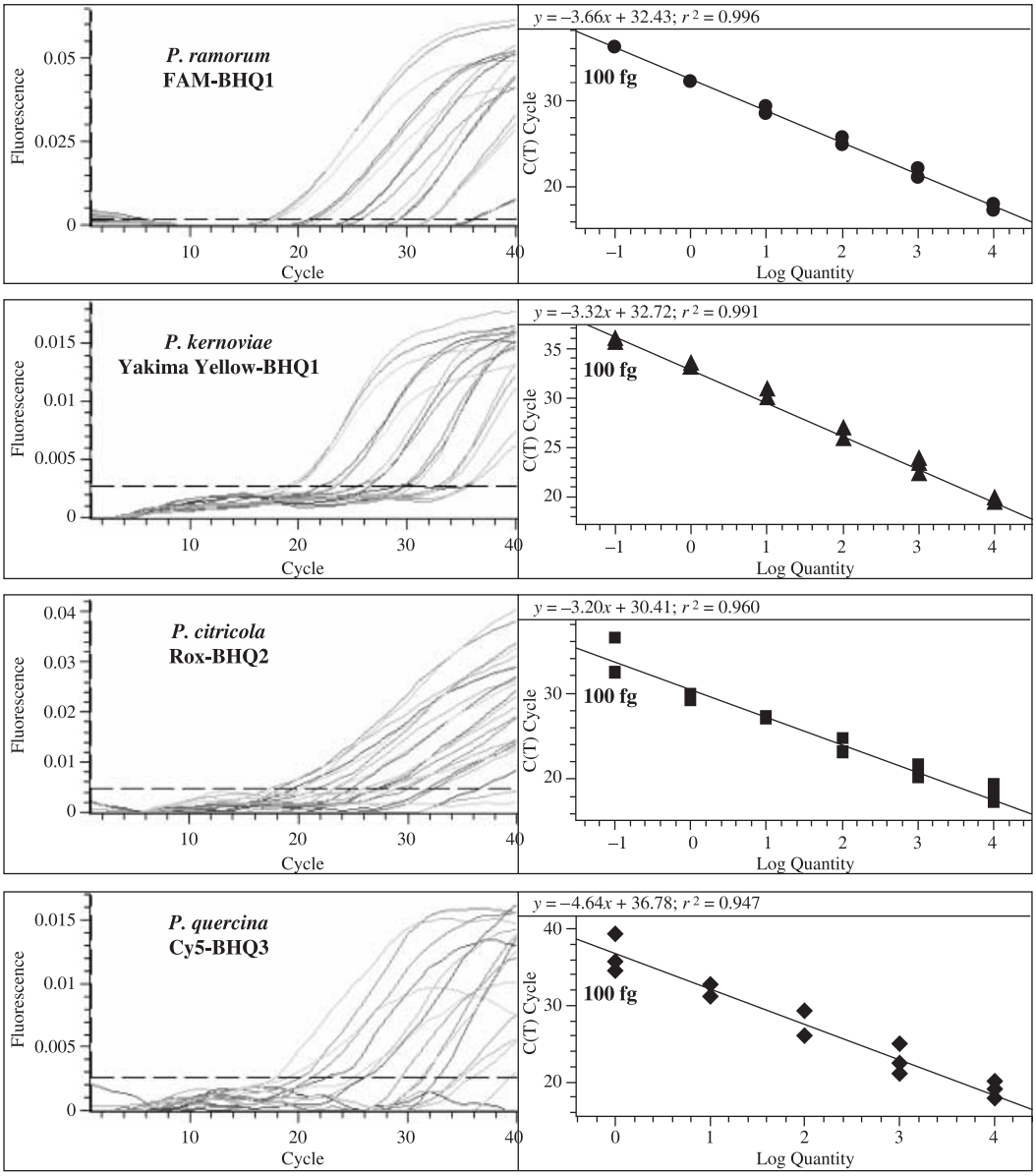

Fig. 3 Detection limits, standard curves and correlation coefficients assessed for *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina*. Total DNA from the four target pathogens was mixed together and serially diluted in water to yield final concentrations ranging from 1 ng/μL to 1 fg/μL before multiplex real-time PCR amplification.

experiments were carried out in which dilution series were performed using extracted DNA from decaying oak leaves instead of water. Decaying oak leaves were previously tested to verify the absence of any *Phytophthora* infection. The presence of plant DNA and coextracted compounds did not significantly affect the amplification of target DNA, although the variance of some measurements was slightly increased when target DNA was diluted in extracted material from decaying oak leaves. Ct values and levels of fluorescence were virtually identical to those obtained when diluting DNA in water (data not shown).

Test with artificially inoculated samples

Artificially infected samples were obtained by inoculating leaves of rhododendron (*R. ponticum*), oak (*Q. ilex*), beech (*F. sylvatica*), sweet cherry (*P. avium*) and birch (*B. pendula*) with *P. ramorum*, *P. kernoviae*, *P. citricola* or *P. quercina*. After inoculation and incubation in a moist chamber for 7–15 days all pathogens infected and colonized leaves of oak, cherry and beech. Only *P. kernoviae* and *P. ramorum* where able to colonize rhododendron leaves and only *P. citricola* and *P. ramorum*

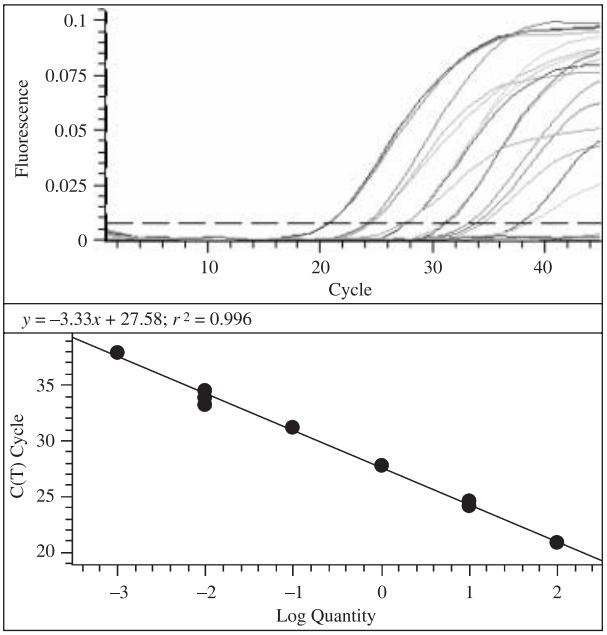

Fig. 4 Detection limits, standard curves and correlation coefficients assessed for *Phytophthora ramorum* in non-multiplex PCR, i.e. in a reaction with only *P. ramorum* DNA and with only the specific primers and probe for this species. Sensitivity and correlation coefficients were identical to those obtained in multiplex PCR (Fig. 3). Similar results were obtained for the other three target pathogens *P. kernoviae*, *P. citricola* and *P. quercina*, i.e. the level of sensitivity and correlation coefficients were largely identical to those obtained in multiplex PCR.

colonized the leaves of birch. Leaf tissues from all host–pathogen combinations with evident symptoms of disease were subjected to DNA extraction and analysed with the universal primers and probe and by multiplex real-time PCR assay. The universal primers and probe amplified DNA from all samples including the uninoculated control leaves with Ct values ranging from 11.1 to 18.2 (Table 3). The specific multiplex real-time PCR enabled the detection of all target pathogens in the infected leaves (Table 3). No increase in fluorescence was obtained in any of the uninoculated control leaves.

Twelve amplicons (three per each pathogen) were randomly picked from positive samples and sequenced according to Schena and Cooke (2006) to confirm that the correct sequence was amplified.

Analysis of naturally infected samples

DNA samples from 77 naturally infected plant hosts were tested during a visit to the Central Science Laboratory (CSL, Sand Hutton, York, UK) and amplified by real-time PCR with the universal primers and probe and by multiplex real-time PCR with the specific primers and probes for *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina*. DNA had been extracted at CSL following the protocol reported by Hughes *et al.* (2006). All samples had been previously analysed using a TaqMan-based method to detect *P. ramorum* and by traditional isolation of *Phytophthora* species on nutritive media (Hughes *et al.*, 2006). The multiplex real-time PCR assays were conducted in a blind experiment, with results of previous molecular analysis and culturing unknown until PCR analyses were completed.

The analyses of naturally infected samples showed the presence of *P. ramorum* and *P. kernoviae* infections in 42.8 and 18.2% of analysed samples, respectively (Table 4). No combined infections of *P. ramorum* and *P. kernoviae* were found. *P. citricola* was detected in a single sample whereas *P. quercina* was not detected in any of the analysed samples. The same results had been previously obtained by traditional isolation on selective media with the only exception being a negative sample in molecular analysis from which *P. kernoviae* had been previously isolated although at a very low level of infection (Table 4). Furthermore, a single sample was positive for *P. ramorum* using the multiplex real-time PCR approach and by traditional isolation, which in a previous analysis with the TaqMan-based method of Hughes *et al.* (2006) had tested negative.

The use of the universal probe and primers confirmed that all extracted DNA was amplifiable and of good quality; Ct values ranged from 13.8 to 19.1.

Ten amplicons (five for *P. ramorum* and five for *P. kernoviae*) were picked at random from positive samples and sequenced according to Schena and Cooke (2006) to confirm that the correct sequence was amplified.

DISCUSSION

In the present paper we have described a TaqMan-based method for the detection and quantification of *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in symptomatic leaves. The method is based on real-time PCR with two universal primers and a probe to monitor the quality of DNA extracted from environmental samples followed by a multiplex real-time PCR approach for the simultaneous detection of the four target pathogens. Two *Phytophthora*-specific primers (Yph1F–Yph2R) were also designed and utilized in a nested approach with the aim of improving the sensitivity of the test. All primers and probes proved effective: (i) the universal primers and probes produced an increase in fluorescence from all tested DNA samples, including *Phytophthora*, *Pythium* and leaf extracts; (ii) the species-specific primers and probes yielded an increase in fluorescence exclusively from many isolates of each target species, but not from any other *Phytophthora* or *Pythium* species tested; and (iii) the *Phytophthora*-specific primers amplified a fragment of the expected size from all species of *Phytophthora* (Table 1), but not from any of the *Pythium* species tested. The universal primers and the probe were designed in a conserved region of the 18S gene and proved very effective as a test of extracted DNA quality (amplifiability)

Table 3 Real-time multiplex PCR detection of *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in artificially inoculated leaves of oak, rhododendron, cherry, beech and birch. Two universal primers and a FAM-labelled probe were utilized to confirm the extraction of amplifiable DNA from infected and non-infected leaves.

		Cycle thresholds				
Inoculated species	Inoculated pathogens	*P. ramorum* (FAM)*	*P. kernoviae* (YY)*	*P. citricola* (Rox)*	*P. quercina* (Cy5)*	Universal Probe (FAM)*
Oak	Uninoculated	—	—	—	—	12.7
	P. citricola	—	—	24.2	—	13.5
	P. kernoviae	—	22.6	—	—	12.9
	P. quercina	—	—	—	27.1	13.2
	P. ramorum	21.8	—	—	—	12.9
Rhododendron	Uninoculated	—	—	—	—	18.2
	P. kernoviae	—	26.2	—	—	18.1
	P. ramorum	23.1	—	—	—	16.8
Cherry	Uninoculated	—	—	—	—	13.2
	P. citricola	—	—	25.9	—	13.3
	P. kernoviae	—	28.2	—	—	12.0
	P. quercina	—	—	—	34.8	12.6
	P. ramorum	23.5	—	—	—	13.0
Beech	Uninoculated	—	—	—	—	11.1
	P. citricola	—	—	23.8	—	9.0
	P. kernoviae	—	24.9	—	—	9.9
	P. quercina	—	—	—	27.3	12.6
	P. ramorum	24.3	—	—	—	9.2
Birch	Uninoculated	—	—	—	—	13.8
	P. citricola	—	—	24.2	—	14.1
	P. ramorum	30.2	—	—	—	12.2

*Fluorophores utilized to label the different probes.

Table 4 Detection of *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in naturally infected leaves and comparison of results from real-time multiplex PCR and traditional isolation into culture.

	P. ramorum			*P. kernoviae*			*P. citricola*			*P. quercina*		
	(+)	(–)	(Tot)	(+)	(–)	(Tot)	(+)	(–)	(Tot)	(+)	(–)	(Tot)
Molecular detection	33	44	77	14	63	77	1	76	77	0	77	77
Isolation into culture	33	44	77	15	62	77	1	76	77	0	77	77

(+) = number of positive samples analysed; (–) = number of negative samples analysed; (Tot) = total number of samples analysed.

and quantity. False negatives can occur in PCR-based detection methods; a variety of naturally occurring compounds, such as humic acids, tannins and lignin-associated compounds, can interfere with PCR reactions and inhibit the amplification (Bridge and Spooner, 2001; Cullen and Hirsch, 1998). Therefore a method for the prior assessment of DNA quality is essential despite recent improvements in DNA extraction methods (Bridge and Spooner, 2001; Cullen and Hirsch, 1998). This aspect is particularly important for quarantine pathogens such as *P. ramorum* and *P. kernoviae*, for which results of a molecular analysis could impact upon large-scale eradication schemes or trade. Because there are only four fluorescent markers that can be used at a time, in the present study it was preferred to detect simultaneously as many pathogens as possible and therefore the universal primers and probe were used in a separate preliminary amplification. From a practical standpoint of assaying samples from the field this means a second amplification is required for each sample, which is not time efficient. Considering that *P. ramorum* and *P. kernoviae* are under strict quarantine restrictions, in future applications it might be worthwhile testing their detection alongside the universal primers/probe in a single multiplex amplification. The substitution of the primers and probes for one of the other two pathogens (*P. citricola* or *P. quercina*) with those of a suitably labelled universal marker should be relatively straightforward.

Species- and genera-specific primers developed on a portion of the *Ypt*1 gene (Chen and Roxby, 1996). The appropriateness of

this gene to develop specific detection methods for *Phytophthora* species has been recently reported by Schena and Cooke (2006); however, to our knowledge this is the first report of the practical utilization of the *Ypt*1 gene to develop conventional and real-time PCR detection methods. Alignment of the *Ypt*1 gene sequences reported by Schena and Cooke (2006) and obtained during this study revealed the presence of conserved coding regions flanking very variable introns appropriate for differentiating closely related species such as *P. ramorum* and *P. lateralis*. Importantly, the high interspecific genetic variability was accompanied by very low levels of intraspecific variation that could cause problems for diagnostic assays. A few polymorphic nucleotides were identified between European and American isolates of *P. ramorum*; however, the selected primers and probes were designed to detect both populations.

Tests to assess the sensitivity of the multiplex detection system showed a detection limit of 100 fg. Higher detection limits have been reported for a range of plant pathogens especially with primers developed on multicopy genes such as the ITS regions of the rDNA (Hayden *et al.*, 2004; Ippolito *et al.*, 2004). Our result was expected because unlike assays designed on rDNA, the *Ypt*1 gene is a single copy gene (Chen and Roxby, 1996). In the current study a nested approach based on a first round amplification with genus-specific primers and a second amplification with species-specific multiplex real-time PCR was developed to improve the level of sensitivity. However, surprisingly, the nested assay was not more sensitive. In nested PCR, a very early increase in fluorescence (average Ct 19.5) was observed for the amplification mixture containing 100 fg of target DNA, but no amplification occurred at any lower DNA concentration, suggesting that no more DNA was available for amplification. This result is consistent with the fact that single copies of target DNA are being amplified by a single round of PCR and thus the nested assay only improved the signal strength without increasing sensitivity. This hypothesis is confirmed by the total content of DNA per nucleus of related oomycetes such as *Achlya* spp. (40 fg), *Saprolegna* spp. (200 fg), *Albugo* spp. (47–80 fg), *Bremia* spp. (72–147 fg), *Paraperonospora* spp. (152–144 fg), *Peronospora* spp. (47–138 fg), *Plasmopara* spp. (92–163 fg) and *Pseudoperonospora* spp. (82–90 fg) (Tanaka *et al.*, 1982; Voglmayr and Greilhuber, 1998). Furthermore, if we consider that rDNA genes occur in multiple copies (414 ± 12 copies per haploid genome in *P. infestans*) (Judelson and Randall, 1998) it is possible to explain their higher levels of sensitivity. From a practical point of view these data indicate that the lower level of sensitivity achieved with the *Ypt*1 gene is a minor problem for this real-time assay as even single copies of the gene and therefore even single propagules of target *Phytophthora* species could be detected by a single multiplex real-time PCR amplification. Furthermore, methods based on single copy genes are not affected by the number of tandem repeats as in multicopy genes and there is the potential to correlate Ct values accurately with the pathogen biomass and/or with the number of propagules. On the basis of these results, the nested approach was not utilized during the present study to detect target species in infected leaves. The *Phytophthora* generic primers, however, do have practical applications for the non-specific detection of *Phytophthora* species, or in a nested approach based on conventional PCR, which is known to be less sensitive than real-time PCR (Cullen *et al.*, 2001; Lees *et al.*, 2002).

Using serial dilutions of target DNA, linear responses and high correlation coefficients between the amount of DNA and cycle thresholds for all target pathogens were achieved. Furthermore, detection limits and correlation coefficients were not affected by the presence of plant extracts. These results indicate that the method developed herein is appropriate for both qualitative and quantitative analyses of pathogen biomass in infected tissues. In particular, the method utilized to extract DNA from lyophilized infected leaves is based on the physical disruption of cells within the samples to produce a high yield of DNA and on the subsequent removal of coextracted compounds by the use of PVPP spin column chromatography (Schena and Ippolito, 2003). The protocol has a small number of lysis and purification steps and maximizes the yield and quality of recovered DNA, although the requirement for lyophilized tissues and the use of organic solvents could reduce its suitability for large-scale assays especially when conducted by regulatory diagnostic laboratories. Lyophilized samples can be easily stored and their DNA extracted after several days, and the same protocol is also appropriate for non-lyophilized samples (Schena and Ippolito, 2003). Organic solvents are widely used in a large number of leading and secondary laboratories because protocols based on these solvents are usually very effective and less expensive than commercial kits. Regardless, the multiplex approach developed in the present study also effectively amplified DNA from naturally infected leaves which was obtained without organic solvents using a NucleoSpin Plant extraction kit (Macherey-Nagel, Düren, Germany) (Hughes *et al.*, 2006).

Comparative testing with field samples showed that the multiplex real-time PCR method developed gave comparable results to the pathogen isolation technique, with only a single discrepancy in a sample that was positive by isolation and negative by real-time PCR. It is difficult to confirm completely the infection status of this sample, but it seems most likely that the positive isolation result is correct and that by chance a non-infected portion of the leaf was selected for DNA extraction. Current methods to detect *Phytophthora* species from plant material are mainly based on the isolation of the pathogens on semi-selective media followed by morphological identification. Such methods, however, are time consuming and result in a 6- to 9-day lag between sample arrival in the laboratory and results being available. Methods based on PCR present clear advantages, allowing the direct detection of pathogen from plant material and providing

results in one or two working days. Increasingly such methods are being used to validate conventional methods with the objective of replacing them. PCR detection methods based on conventional PCR have been developed for a large number of *Phytophthora* species including *P. ramorum* (Hayden *et al.*, 2004; Martin *et al.*, 2004), *P. citricola* and *P. quercina* (Nechwatal *et al.*, 2001; Schubert *et al.*, 1999). These methods, however, require the time-consuming step of gel electrophoresis, use chemicals not suited to routine diagnostic work (e.g. ethidium bromide) and, being open tube systems, are potentially more susceptible to PCR contamination. A detection method based on SYBR Green has been recently published for *P. ramorum* (Hayden *et al.*, 2004). However, although the method is an improvement on conventional PCR, it still relies on a nested-PCR approach, which is more time consuming and could be prone to contamination problems. Furthermore, SYBR Green is a sequence-non-specific method and therefore less specific compared with alternative methods such as TaqMan (Mackay *et al.*, 2002). Amplicon sequence-specific methods based on TaqMan have been developed for *P. ramorum* using as target genes the ITS1 region (Hughes *et al.*, 2006; Tomlinson *et al.*, 2005). Because ITS regions of *P. ramorum* are very similar to those of *P. lateralis* (Werres *et al.*, 2001), in both methods it was necessary to introduce a base substitution in one of the two primers to increase specificity. This did, however, result in a decrease in sensitivity (Hughes *et al.*, 2006). In a comparative analysis using leaves naturally infected by *P. ramorum* the method developed by Hughes *et al.* (2006) provided the same results as the multiplex real-time PCR approach developed in the present study, although Ct values were always higher by one or two orders in the ITS-based method (L.S. and K.J.D.H., unpublished data). A slightly reduced sensitivity of the method of Hughes *et al.* (2006) was also confirmed by the identification of a single sample that was negative to *P. ramorum* using the Hughes method but positive with both the multiplex real-time PCR approach and traditional isolation. Recently, a multiplex PCR method for the detection of *P. ramorum* and *P. pseudosyringae* has been developed on a portion of the mitochondrial DNA with a very high AT/GC ratio (Tooley *et al.*, 2006). In that study the simultaneous detection of both pathogens caused a significant reduction of sensitivity compared with separate reactions.

One of the main goals of the present research was the development of a very specific multiplex approach to detect simultaneously four target pathogens without any reduction of sensitivity compared with single amplifications. To obtain this result, several factors were optimized, including primer and probe design, amplification conditions and length of the amplified fragments. The last aspect played a major role. Primers reported in the present paper were designed to amplify very short PCR fragments (only 1–3 bases separated primers from probes), whereas in previous unreported preliminary tests the amplification of PCR fragments ranging from 100 to 180 bp caused a reduction of sensitivity in multiplex PCR compared with separate PCR reactions (L.S. and D.E.L.C., unpublished data). Furthermore, considering the complexity of the reaction (eight different primers and four different probes were utilized together) it was essential that BHQ were used that dissipate energy as heat to avoid overlapping of fluorescence emission peaks from fluorophores and quenchers.

In conclusion, for the first time in plant pathology, we describe a multiplex real-time PCR method to detect and quantify four important target pathogens. This approach is an advancement on previous reports aimed at the detection of single pathogens and should facilitate the development of similar methods with other targets. Our method proved to be rapid, reliable, sensitive and cost effective; multiple pathogens can be detected within the same plant extract by using different primer/probe combinations. The present approach has great potential as a tool for identification and detection in a range of applications from pathogen surveys to statutory testing. The need for such a test is especially pressing in light of the recent spread of *P. ramorum* in the USA and Europe, the threat represented by the possible dissemination of *P. kernoviae* and the wide distribution of *P. citricola* and *P. quercina* in Europe.

EXPERIMENTAL PROCEDURES

Phytophthora isolates and DNA extractions

Seventy-two isolates (35 *Phytophthora* species) sourced from the culture collections of the authors and from CABI Biosciences (Egham, UK) were used (Table 1). Emphasis was put on species known to be forest pathogens. Isolates were stored on oatmeal agar at 5 °C and grown on French bean agar for routine stock cultures.

The procedure reported by Schena and Cooke (2006) was utilized to extract DNA from pure cultures of *Phytophthora* spp., whereas to extract DNA from naturally infected and artificially inoculated leaves a slightly modified version of the procedure described by Schena and Ippolito (2003) was used. Approximately 10 mg of freeze-dried leaf tissues was transferred to 2-mL screw-cap tubes containing an equal volume of polyvinylpyrrolidone (PVP) together with 5-mm stainless steel ball bearings, 0.2 g each of 0.1-mm-diameter zirconia/silica beads, 1.0-mm-diameter glass beads and 1.5 mL of extraction buffer [200 mM Tri-HCl (pH 7.7), 250 mM NaCl, 25 mM EDTA, 0.5% SDS]. The extraction mixture was blended in a Mini-BeadBeater (Bio-Spec Products, Bartlesville, OK, USA) at 5000 r.p.m. for 60 s and centrifuged at 13 000 ***g*** for 5 min. After centrifugation the upper phase (approximately 800 μL) was extracted twice with 1 mL of phenol/chloroform (1 : 1) and 700 μL of chloroform, respectively. DNA was precipitated with an equal volume of isopropanol for 1 h at 5 °C,

washed with 70% cold ethanol (–20 °C), dried and resuspended in 100 μL of sterile distilled water (Sambrook *et al.*, 1989).

Before amplification, DNA extracted from leaves was purified using polyvinylpolypyrrolidone (PVPP) spin columns as described by Schena and Ippolito (2003). All DNA samples were kept at –20 °C for long-term storage and at 5 °C for routine amplifications.

Primer and probe design

Specific primers and probes to identify *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* were designed in different regions of the *Ypt*1 gene and in particular in introns 3 (*P. ramorum*), 4 (*P. kernoviae*) and 5 (*P. citricola* and *P. quercina*) (Chen and Roxby, 1996) (Fig. 1, Table 2). All sequences of the *Ypt*1 gene reported by Schena and Cooke (2006) plus an additional 27 sequences obtained during this study (Table 1) were utilized as target to design molecular markers. Additional sequences were amplified using primers Ypt1F–Ypt2R (Fig. 1, Table 2) and sequenced following the procedure described by Schena and Cooke (2006). A total of 70 sequences from 70 different isolates representative of 35 different *Phytophthora* species were aligned using the MultAlin software (Corpet, 1988) and screened for differences among species. Primers and probes were designed using the Primer express™ 1.5 software (Applied Biosystems) and purchased from Eurogentec Ltd (Begium). All primers were designed to have a melting temperature of 60 ± 1 °C and combined with probes having a melting temperature 10 °C higher (70 ± 1 °C). Specific probes were labelled with FAM (*P. ramorum*), Yakima Yellow® (*P. kernoviae*), Rox (*P. citricola*) and Cy®5 (*P. quercina*) to allow the simultaneous detection of the four pathogens in a single reaction (multiplex PCR). BHQ that dissipate energy received from fluorophores as heat were utilized to prevent any interference among fluorescence emission spectra of fluorophores and quenchers (Eurogentec). Fluorophores FAM and Yakima Yellow were combined with the quencher BHQ1, whereas the fluorophores Rox and Cy5 were combined with the quenchers BHQ2 and BHQ3, respectively.

Two conventional primers (Yph1F–Yph2R) were designed in the two coding regions flanking introns 3, 4 and 5 to amplify this region from all *Phytophthora* species (Fig. 1, Table 2). To this aim all sequences from *Phytophthora* spp. were aligned with the same portion of the gene amplified and sequenced from 12 *Pythium* species (Moorman *et al.*, 2002). The localization of primers Yph1F–Yph2R enabled the development of a nested PCR, providing a first common amplification with primers Yph1F–Yph2R and a second amplification by real-time multiplex PCR.

Two universal primers (LS-F and LS-R) and a universal probe (LS-P) were designed using the approach described above in a conserved region of the 18S region of the rRNA gene to amplify DNA from a wide range of organisms including *Streptophyta*, *Fungi* and *Stramenopiles*. The universal probe was labelled with FAM and quenched with BHQ1 (Table 2).

Amplification conditions

All real-time PCR reactions were performed in a total volume of 15 μL containing 1 μL of genomic DNA (concentration of 10 ng/μL in specificity tests), 1.5 μL of 10× Reaction Buffer (qPCR™ Core Kit, Eurogentec, Belgium), 5 mM $MgCl_2$, 200 μM each dNTPs, 0.5 unit of *Taq* polymerase (qPCR Core Kit) and 0.33 μM of each primer and 0.13 μM of each probe. PCR amplification conditions consisted of one cycle of denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 20 s and 62.5 °C for 20 s. Fluorescence was monitored at each PCR cycle during the annealing–extension phase at 62.5 °C. Amplifications were performed using an Chromo 4™ Detector and data acquisition and analysis realized using the supplied Opticon Monitor™ software version 2.03 (MJ Research, Waltham, MA, USA) according to the manufacturer's instructions. The cycle threshold (Ct) values for each reaction were calculated automatically by the Opticon Monitor software by determining the PCR cycle number at which the reporter fluorescence exceeded background.

Conventional PCR reactions were performed in a total volume of 15 μL containing 1 μL of genomic DNA (concentration of 10 ng/μL in specificity tests), 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 100 μM dNTPs, 1 mM $MgCl_2$, 50 μg BSA, 1 unit of *Taq* polymerase (Taq DNA polymerase, Promega Corp., WI, USA) and 6 μM of primers. PCR amplification conditions consisted of: one cycle of 95 °C for 2 min; 35 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C for 30 s; and a final cycle of 72 °C for 10 min. Amplicons were analysed by electrophoresis in 2% agarose gels containing SYBR Safe™ DNA gel stain (Invitrogen, UK) in TBE buffer (Sambrook *et al.*, 1989) and visualized under UV light.

Production of artificially inoculated leaves

Artificially infected leaves were obtained by inoculating 20 leaves of *R. ponticum*, *Q. ilex*, *F. sylvatica*, *P. avium* and *B. pendula* with *P. ramorum* (isolate SCRP911), *P. kernoviae* (isolate SCRP722), *P. citricola* (isolate SCRP130) or *P. quercina* (isolate SCRP541). Tender detached leaves collected in May 2005 were wounded in the middle of the lamina with a needle, inoculated with a plug of mycelium in agar and maintained in moist chambers at 21 °C for 7–15 days until apparent symptoms of decay appeared on the lamina. Twenty non-inoculated wounded leaves were utilized as negative controls. Leaf tissues immediately around the inoculation points were discarded and remaining tissues were freeze-dried for extended storage at –20 °C. For each leaf species and for each pathogen species DNA was extracted in triplicate and 1 μL of purified DNA was amplified as described above.

Naturally infected leaf samples

In this study multiplex real-time PCR was utilized to analyse 77 field samples previously selected across England by Defra's Plant Health and Seeds Inspectorate (PHSI) during an extensive monitoring of *P. ramorum* and *P. kernoviae* (Hughes *et al.*, 2006). Samples were from 11 hosts as follows: 49 *Rhododendron*, six *Pieri*, five *Leucothoe*, eight *Vaccinium*, three *Kalmia*, and individual samples from *Camellia*, *Larus*, *Magnolia*, *Taxus*, *Quercus* and *Sequoia*.

ACKNOWLEDGEMENTS

This work was funded by the European Union with a Marie Curie Intra-European Fellowship (Project: MEIF-CT-2003-502327) and by the Scottish Executive Environment and Rural Affairs Department (SEERAD). We thank Prof. C. M. Brasier at Forest Research Agency, Farnham, UK, for providing isolates, N. Williams for invaluable help with the culture collection and Dr J. M. Duncan and Prof. A. Ippolito for their support in establishing the co-operation between the Department of Plant Protection and Applied Microbiology and the Scottish Crop Research Institute. Cultures and plant materials were held under SASA Licence No. PH/37/2005 at SCRI and Defra Licence No. PHL 251 A/5016/02/2005 at CSL.

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