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Development and application of a PCR-based 'molecular tool box' for the identification of *Phytophthora* species damaging forests and natural ecosystems

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A PCR-based 'molecular tool box', based on a region of the ras-related protein gene *Ypt*1, was developed for the identification of 15 *Phytophthora* species that damage forests and trees: *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citricola*, *P. europaea*, *P. inundata*, *P. lateralis*, *P. megasperma*, *P. nemorosa*, *P. kernoviae*, *P. pseudosyringae*, *P. psychrophila*, *P. quercina*, *P. ramorum* and *P. ilicis*. Most primers proved highly specific in BLAST analyses and in tests with DNA from 72 isolates of 35 species of *Phytophthora* and nine species representative of *Pythium*. Exceptions were primers designed for *P. cactorum* and *P. ilicis*, which cross-reacted with *P. idaei* and *P. nemorosa*, respectively. Amplification with *Phytophthora*-genus-specific primers before amplification with the various species-specific primers (nested PCR) increased the sensitivity of detection over amplification with species-specific primers only: detection limits ranged between 100 and 10 pg target DNA μL^{-1} in the latter, compared with 100 fg μL^{-1} in nested PCR. Using existing methods for rapid extraction and purification of DNA, single-round amplification was appropriate for detection of target *Phytophthora* species in leaves, but nested PCR was required for soil and water samples. The quarantine pathogens *P. ramorum* and *P. kernoviae* were detected in a number of naturally infected leaves collected in England and Wales, whereas *P. citricola* was commonest in water and soil samples from natural Scottish ecosystems.

Keywords: forests and natural ecosystems, molecular tool box, *Phytophthora* spp., ras-related protein gene, *Ypt*1 gene

Introduction

The oomycete genus *Phytophthora* comprises more than 70 different species of plant pathogens, many responsible for some of the most serious and economically important plant diseases. Currently, approximately 15 species are recognized as posing a considerable threat to forest and natural ecosystems; some have been known for many years ('old' species), but many are 'new' taxa, only isolated and characterized within the last 10 years (Cooke *et al*., 2007). 'Old' and 'new' are frequently found in 'clusters' on the same sites, sometimes even the same tree (Vettraino *et al*., 2002, 2005).

The host and geographical ranges of many of these species are considerable. For example, among the most frequent and damaging of the 'old' species is *P. cinnamomi*, the cause of Jarrah dieback in Australia, in which trees of the dominant and economically important *Eucalyptus marginata,* as well as many understorey plants, such as *Banksia*, are damaged or destroyed (Jurskis, 2005). In the USA and Europe, *P. cinnamomi*, together with *P. cambivora*, causes ink disease of sweet chestnut, currently spreading aggressively in most chestnut-growing areas of Italy and thought to be a major factor in the mortality of *Castanea sativa* (Vettraino *et al*., 2005). *Phytophthora cinnamomi* has also been implicated in the severe decline of oaks in Europe (Luque *et al*., 2002). Likewise, in Europe, *P. citricola* has been isolated from a wide range of economically important hosts; it was the commonest species found on *Fagus sylvatica* (beech), from which *P. cambivora* and *P. cactorum* can also be recovered. In contrast, in the USA, *P. inflata*, very closely related to *P. citricola*, was the most important species on beech (Jung *et al*., 2005).

The role of less commonly encountered 'old' species, such as *P. cryptogea, P. megasperma*, *P. syringae*, *P. ilicis*, *P. drechsleri* and *P. gonapodyides* in declines of trees and natural ecosystems is debatable. However, pathogenicity tests suggest that all *Phytophthora* spp. could be significant in such declines, especially when different species are present in a cluster and conditions are favourable for the development of disease (Jung *et al*., 2005).

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Examples of 'new' species that pose a considerable threat to forests and natural ecosystems are *P. ramorum*, *P. kernoviae*, *P. alni* and *P. quercina*. *Phytophthora ramorum* has destroyed large areas of native Californian oak forest, killing oaks and other trees from a range of genera (Rizzo *et al*., 2005). It also causes leaf blights or dieback on understorey shrubs and ornamental nursery stock such as rhododendron (Tooley *et al*., 2004). In Europe, *P. ramorum* has been isolated frequently from *Rhododendron* and other shrubs in nurseries and recently from a number of trees (Brasier *et al*., 2004a). *Phytophthora kernoviae* causes bleeding stem lesions on beech (Brasier *et al*., 2005; Brown & Brasier, 2007) and *P. alni* (Brasier *et al*., 2004b) has killed thousands of alders across a broad swathe of Central and Northern Europe. The latter represents a threat to natural and managed alder stands in Europe and the stability of associated riparian ecosystems. Further afield, *P. alni*, which appears to be the product of one or more interspecific hybridization events (Brasier *et al*., 1999), could represent a threat to alder populations in Asia and North America. Very aggressive to fine roots of oaks and implicated in the rapid decline of oaks growing on acid, well-drained soils, *P. quercina* is one the most widespread and most frequently isolated species across Europe (Jung *et al*., 2000; Cooke *et al*., 2005).

Six other 'new' species isolated recently from important trees are *P. uliginosa* and *P. europaea* (from oak), *P. pseudosyringae* (oak and beech), *P. psychrophila* (oak), *P. inundata* (several hosts) and *P. nemorosa* (several hosts) (Brasier *et al*., 2003; Jung *et al*., 2002, 2003; Hansen *et al*., 2003).

The discovery of so many *Phytophthora* species in such a short time (~10 years), is in part attributable to improved detection methods, but other factors are probably involved, including climate change (Coakley *et al*., 1999; Rizzo *et al*., 2005) and increased movement of the pathogens in plant material across Europe, e.g. in woody ornamental plants produced in large nurseries growing many different plants for international wholesale trade.

PCR, conventional and real-time, has emerged as an important tool for the diagnosis and study of phytopathogenic fungi and has solved some of the problems associated with their detection, control and containment (Schena *et al*., 2004). Further improvements of PCR-based diagnostics (PCRDs) would expand knowledge of the occurrence, population biology and genetics of phytophthoras damaging to trees and natural ecosystems. Currently, PCRDs are available for some *Phytophthora* species which are known to cause diseases in forest trees (Table 1). Most of these PCRDs are based on the internal transcribed spacer (ITS) regions and on sequence-characterized amplified regions (SCAR). However, ITS sequences are not always sufficiently variable to separate closely related taxa (Kroon *et al*., 2004; Schena & Cooke,

Table 1 Updated list of primers forward (F) and reverse (R) reported for the identification and detection of *Phytophthora* species known to threaten forests and other natural ecosystems

Phytophthora species	Primer F	Primer R	Target	Chemistry	Reference
Alder Phytophthoras	D16F	D16R	SCAR	Conventional PCR	De Merlier *et al.* (2005)
P. alni subsp. *alni*	PA-F	PA-R	SCAR	Conventional PCR	Ioos *et al.* (2005)
P. alni subsp. *multiformis*	PAM-F	PAM-R	SCAR	Conventional PCR	Ioos *et al.* (2005)
P. alni subsp. *uniformis*	PAU-F	PAU-R	SCAR	Conventional PCR	Ioos *et al.* (2005)
P. cactorum	PC1	PC2	SCAR	Conventional PCR	Causin *et al.* (2005)
P. cambivora	CAMB3	CAMB4	RAPD	Conventional PCR	Schubert *et al.* (1999)
P. cinnamomi	95·422	96·007	*Cina-6a* gene	Other[a]	Coelho *et al.* (1997)
	LPV2 F	LPV2 R	*Lpv* gene	Conventional PCR	Kong *et al.* (2003)
	LPV3 F	LPV3 R	*Lpv* gene	Conventional PCR	Kong *et al.* (2003)
P. citricola	CITR1	CITR2	ITS region	Conventional PCR	Schubert *et al.* (1999)
	P5	P6	ITS region	TaqMan	Böhm *et al.* (1999)
	Ycit3F	Ycit4R	*Ypt*1	TaqMan	Schena *et al.* (2006)
P. kernoviae	Yptc3F	Yptc4R	*Ypt*1	TaqMan	Schena *et al.* (2006)
P. lateralis	Platf	Platr	ITS region	Conventional PCR	Winton & Hansen (2001)
P. nemorosa	FMnem-1	FMnem-3	*Cox2-Cox1* gene	Conventional PCR	Martin *et al.* (2004)
P. pseudosyringae	FMPps1c	FMPps2c	mtDNA	TaqMan	Tooley *et al.* (2006)
	FMPps1c	FMPps2c	*Cox2-Cox1* gene	Conventional PCR	Martin *et al.* (2004)
P. quercina	QUERC1	QUERC2	SCAR	Conventional PCR	Schubert *et al.* (1999)
	QUERC3	QUERC4	SCAR	Conventional PCR	Nechwatal *et al.* (2001)
	Yque3F	Yque4R	*Ypt*1	TaqMan	Schena *et al.* (2006)
P. ramorum	Phyto1	Phyto4	ITS region	SYBR Green	Hayden *et al.* (2004)
	FMPr-1a	FMPr-7	*Cox2-Cox1* gene	Conventional PCR	Martin *et al.* (2004)
	Pram-114Fc	*Pram*-190R	ITS region	TaqMan	Hughes *et al.* (2006)
	FMPr-1a	FMPr-7	mtDNA	TaqMan	Tooley *et al.* (2006)
	Yram4F	Yram3R	*Ypt*1	TaqMan	Schena *et al.* (2006)

[a]Colorimetric assay involving an oligonucleotide capture probe covalently immobilized on microtitration wells, a multi-biotinylated oligonucleotide detection probe and the PCR-amplified target DNA.

2006; Schena *et al*., 2006) and the development of SCAR primers is very laborious (Schena *et al*., 2004). Furthermore, PCRDs reported in Table 1 are geared to the detection of particular species and are therefore not ideal for determining all *Phytophthora* species that might be present in 'clusters' in natural ecosystems.

Among alternative target genes proposed as the basis of PCRDs, the ras-related protein gene *Ypt*1 (Chen & Roxby, 1996) possesses conserved exons and very variable introns suitable for the development of PCRDs for almost all *Phytophthora* species (Schena & Cooke, 2006). This gene was used to develop a pair of *Phytophthora*-genus specific primers (Yph1F-Yph2R), as well as a multiplex real-time PCR approach to detect and quantify *P. ramorum, P. kernoviae, P. citricola* and *P. quercina* in naturally and artificially infected leaves (Schena *et al*., 2006).

The aim of the present study was to develop and validate a PCRD 'molecular tool box' for the identification of 15 important *Phytophthora* species found in forests and other natural ecosystems. All specific primers were designed based on regions from the *Ypt*1 gene and combined with the genus-specific primers Yph1F-Yph2R to develop a nested PCR approach.

Materials and methods

Phytophthora and *Pythium* isolates

Isolates used in the present study were those reported in Schena *et al*. (2006) and were representative of 35 species of *Phytophthora* (Table 2) and nine species of *Pythium* (*P. pyrilobum* IMI 308312, *P. catenulatum* IMI 323121, *P. torulosum* IMI 308268, *P. intermedium* Py4, *P. dissotocum* IMI 329003, *P. aphanidermatum* Py7, *P. ultimum* Py8, *P. undulatum* IMI 337230 and *P. splendens* IMI 391319).

DNA extraction from pure cultures, leaves, soil and water

DNA was extracted from pure cultures of *Phytophthora* and *Pythium* (Schena & Cooke, 2006), naturally and artificially infected leaves (Schena *et al*., 2006) and from naturally infested soil samples (Cullen *et al*., 2001). For DNA from water samples, 10 L water were centrifuged for 5 min at 10 000 ***g*** and pellets (approximately 2 mL) were processed in the same manner as soil samples.

DNA extracted from leaves, soils and water samples was purified using a polyvinylpolypyrrolidone (PVPP) spin column following the procedure described by Cullen *et al*. (2001) and kept at –20°C for long-term storage and at 5°C for routine amplification. Before amplification with specific primers, 1 μL of each DNA sample was amplified in real-time PCR using the universal primers and probe (TaqMan) described by Schena *et al*. (2006) to estimate the quality and quantity of extracted DNA.

Design of specific primers

Potentially specific primers for 15 different species of *Phytophthora* (*P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citricola*, *P. europaea*, *P. inundata*, *P. lateralis*, *P. megasperma*, *P. nemorosa*, *P. kernoviae*, *P. pseudosyringae*, *P. psychrophila*, *P. quercina*, *P. ramorum* and *P. ilicis*) were designed from regions of the *Ypt*1 gene comprising introns 3, 4 and 5 (Fig. 1). Initially, *Ypt*1 sequences from 71 isolates, representing 35 species of *Phytophthora* (Table 2), were aligned using the CLUSTALW software (EMBL, European Bioinformatics Institute) and screened for exploitable differences among species. Using the PRIMER 3 software (Rozen & Skaletsky, 2000), all primers (Fig. 1; Table 3) were designed to have the same amplification requirements, with a melting temperature between

Table 2 Isolates of *Phytophthora* included in the present study and corresponding GenBank accession numbers for sequences of the ras-related protein (*Ypt*1) gene used as target to develop the PCR-based 'molecular tool box'

		Origin		
Phytophthora species	Isolate number(s)	Host	Country	*Ypt*1 gene
P. alni subsp. *alni*	SCRP2	*Alnus* sp.	UK	DQ162953
	SCRP4	*Alnus* sp.	Germany	DQ270297
	SCRP8	*Alnus* sp.	France	DQ270299
P. alni subsp. *multiformis*	SCRP3	*Alnus* sp.	Netherlands	DQ270307
	SCRP14	*Alnus* sp.	Germany	DQ270298
P. alni subsp. *uniformis*	SCRP10	*Alnus* sp.	Sweden	DQ270301
	SCRP12	*Alnus* sp.	Sweden	DQ270300
P. boehmeriae	SCRP23	*Gossypium hirsutum*	China	DQ270324
P. cactorum	IMI 296524; SCRP27	*Rubus idaeus*	Wales, UK	DQ162960
	SCRP30	*Fragaria* × *ananassa*	Sweden	DQ270309
	SCRP35	*Fragaria* × *ananassa*	Scotland, UK	DQ270308
	SCRP39	*Fragaria* × *ananassa*	Scotland, UK	DQ270311
	SCRP48	*Ribes* sp.	England, UK	DQ270310
P. cambivora	IMI 296831; SCRP67	*Rubus idaeus*	Scotland, UK	DQ162954
	SCRP80	*Castanea sativa*	Italy	DQ162955
	SCRP75	*Fagus* sp.	UK	ND[a]
	SCRP82	*Eucalyptus* sp.	Australia	DQ162956

Table 2 *Continued*

Phytophthora species	Isolate number(s)	Origin		*Ypt*1 gene
		Host	Country	
P. capsici	IMI 352321; SCRP103	*Piper nigrum*	India	DQ162972
P. cinnamomi	CBS270·55; SCRP115	*Chamaecyparis* sp.	Netherlands	DQ162959
	CBS342·72; SCRP118	*Persea gratissima*	California	DQ270317
	SCRP121		Australia	DQ270316
P. citricola	SCRP130	*Rubus idaeus*	Scotland, UK	DQ162968
	SCRP136	Soil	UK	DQ162969
	SCRP140	*Taxus* sp.	UK	DQ162970
	SCRP143	*Quercus robur*	Germany	DQ162971
P. citrophthora	IMI 332632; SCRP179	*Actinidia chinensis*	Chile	DQ162973
P. cryptogea	IMI 045168; SCRP207	*Lycopersicon esculentum*	New Zealand	DQ162987
P. drechsleri	ATCC46724; SCRP232	*Beta vulgaris*	USA	DQ162989
P. erythroseptica	SCRP240	*Solanum tuberosum*	Netherlands	DQ162988
P. europaea	SCRP622	*Quercus robur*	Switzerland	DQ162952
P. fragariae var. *fragariae*	SCRP245	*Fragaria* × *ananassa*	England, UK	DQ162950
	SCRP779	*Fragaria* × *ananassa*	Scotland, UK	DQ270306
P. fragariae var. *rubi*	SCRP249	*Rubus idaeus*	Germany	DQ270305
	SCRP278	*Rubus idaeus*	USA	DQ270304
	SCRP310	*Rubus idaeus*	Sweden	DQ270303
	IMI355974; SCRP333	*Rubus idaeus*	Scotland, UK	DQ162951
	SCRP339	*Rubus idaeus*	France	DQ270302
P. idaei	CBS968·95; SCRP370	*Rubus idaeus*	Scotland, UK	DQ270312
	IMI313727; SCRP371	*Rubus idaeus*	England, UK	DQ270313
	SCRP373	*Rubus idaeus*	England, UK	DQ270314
	SCRP376	*Rubus idaeus*	England, UK	DQ270315
P. ilicis	SCRP377	*Ilex aquilifolium*	UK	DQ162962
	SCRP379	*Ilex aquilifolium*	UK	DQ162963
P. infestans	SC03·26·3·3	*Solanum tuberosum*	Scotland, UK	DQ162961
P. insolita	IMI288805; SCRP385	Soil	Taiwan	DQ162974
P. inundata	IMI389751; SCRP644	*Salix* sp.	UK	DQ162982
	IMI389750; SCRP643	*Aesculus hippocastanum*	UK	DQ162983
	SCRP647	*Vitis* sp.	S. America	DQ162984
	SCRP649	*Alnus glutinosus*	Denmark	DQ162985
P. katsurae	SCRP388			DQ162980
P. kernoviae	SCRP722	*Fagus sylvatica*	England, UK	DQ162975
	SCRP957	*Fagus sylvatica*	England, UK	DQ270322
	SCRP958	*Fagus sylvatica*	England, UK	DQ270321
	KER-CSL			DQ270323
P. lateralis	IMI 040503; SCRP390	*Chamaecyparis* sp.	USA	DQ162991
P. medicaginis	SCRP407	*Medicago* sp.	Iran	DQ162990
P. megasperma	IMI 133317; SCRP435	*Malus sylvestris*	Australia	DQ162986
P. nemorosa	SCRP910			DQ162965
P. nicotianae	IMI 268688; SCRP468	*Citrus* sp.	Trinidad	DQ162981
P. palmivora	SCRP526	*Hevea brasiliensis*	Thailand	ND[a]
P. pistaciae	IMI386658; SCRP533	*Pistacia vera*	Iran	DQ162957
P. pseudosyringae	IMI390500; SCRP674	*Malus pumila*	Italy	DQ162966
	SCRP734	*Fagus sylvatica*	Italy	DQ162967
P. psychrophila	SCRP630	*Quercus ilex*		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, UK	DQ162992
	SCRP954	*Viburnum tinus*	England, UK	DQ270319
	SCRP955	*Lithocarpus densiflorus*	Oregon	DQ270320
	SCRP956	*Quercus agrifolia*	USA	DQ270318
P. sojae	SCRP555	*Glycine max*	USA	DQ162958

[a]ND = not determined.

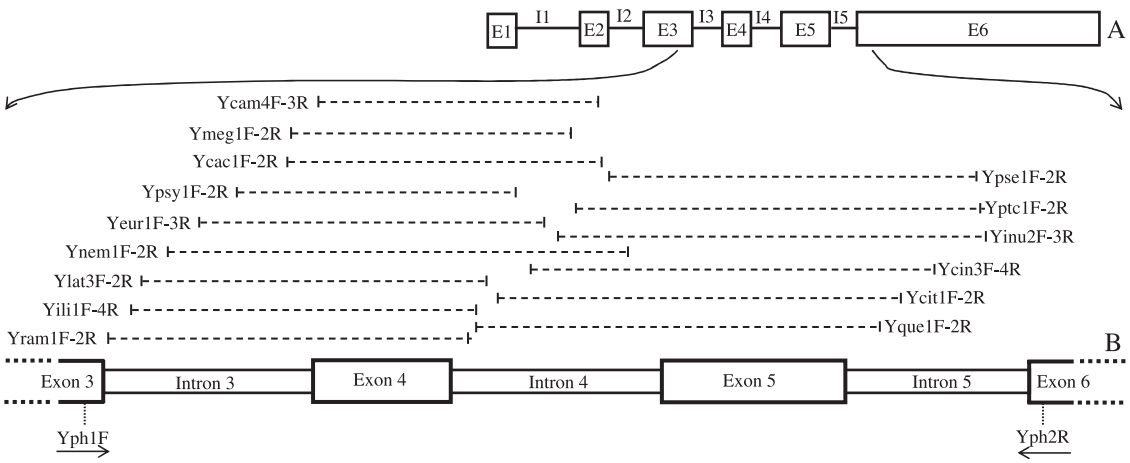

Figure 1 Schematic localization of exons (E) and introns (I) in the ras-related protein (*Ypt*1) gene (Chen & Roxby, 1996) (A) and a more detailed view of the fragment between exons 3 and 6 on which all species-specific primers were designed (B). The developed 'molecular tool box' included species-specific primers for *Phytophthora cambivora* (Ycam4F-3R), *P. megasperma* (Ymeg1F-2R), *P. cactorum* (Ycac1F-2R), *P. psychrophila* (Ypsy1F-2R), *P. europaea* (Yeur1F-3R), *P. nemorosa* (Ynem1F-2R), *P. lateralis* (Ylat3F-2R), *P. ilicis* (Yili1F-4R), *P. ramorum* (Yram1F-2R), *P. pseudosyringae* (Ypse1F-2R), *P. kernoviae* (Yptc1F-2R), *P. inundata* (Yinu2F-3R), *P. cinnamomi* (Ycin3F-4R), *P. citricola* (Ycit1F-2R) and *P. quercina* (Yque1F-2R). *Phytophthora*-genus-specific primers Yph1F and Yph2R where designed on exons 3 and 6, respectively. Dotted lines indicate the region amplified by each pair of species-specific primers, whereas arrows below the genus-specific primers indicate their orientation.

Table 3 *Phytophthora*-genus-specific and species-specific PCR primers generated in this study

Target species	Primer codes and sequences (5′–3′)	AT/CG (%)	TM[a] (°C)	Primer codes and sequences (5′–3′)	AT/CG (%)	TM[a] (°C)
Phytophthora spp.	YPh1F	50·0	63·1	YPh2R	45·0	61·0
	CGACCATKGGTGTGGACTTT	55.0	60·8	ACGTTCTCMCAGGCGTATCT	50·0	57·4
P. cactorum	Ycac1F CCATACAAAATTCTGCGCTAGG	54·5	61·0	Ycac2R AGACACACAAGTGGACCGTTAG	50·0	59·2
P. cambivora	Ycam4F TGGCTAAGTTTTGACCTCCAG	52·3	59·4	Ycam3R ACAATTCCGAATAATCACAGTGTA	66·7	57·7
P. cinnamomi	Ycin3F GTCCTATTCGCCTGTTGGAA	50·0	60·1	Ycin4R GGTTTTCTCTACATAACCATCCTATAA	66·7	57·5
P. citricola	Ycit1F TCCAACTTAGTAAGAGTGCTGGA	56·5	58·2	Ycit2R CAACAGAAATCCTGAAGTACTGTATCA	62·3	60·0
P. europaea	Yeur1F GCCTTGTCTGTCCATGGCTTA	47·6	62·5	Yeur3R AATAATCAAAGCGTACACCAGTT	65·2	57·0
P. ilicis	Yili1F GTGGACTTTGTAAGTGACATCG	50·0	57·3	Yili4R ACAAGTTAGTTAGATGTCCGAGCCATA	59·3	62·5
P. inundata	Yinu2F GGTTTCATGGGCGAAATTAAC	57·1	60·5	Yinu3R CCGAGGTCAACTGGTATAGACG	45·5	60·9
P. kernoviae	Yptc1F AGCTTCTGGGAAGGGCTATG	45·0	60·7	Yptc2R TCATGTGGTGGCAGATAGTTG	52·4	59·6
P. lateralis	Ylat3F ACTGCTGATGACGGGATCG	42·1	62·3	Ylat2R AAAAATCTCCCGCAGACATAC	57·1	58·2
P. megasperma	Ymeg1F TCTGCTCTTCCGACTTGGTC	45·0	60·5	Ymeg2R TGGCATTAGTTAGTTTCGTCCA	59·1	59·7
P. nemorosa	Ynem1F CGCCTTTGAGGGTAGGGAAT	45·0	62·5	Ynem2R CACACGTGAATACCCCAACA	50·0	60·3
P. pseudosyringae	Ypse1F AACTTGGTGCGGTAATCACG	50·0	60·9	Ypse2R GGCCTCTTCGGTAACCCTAC	40·0	60·0
P. psychrophila	Ypsy1F CTTCGAGGGCAGGAAAGG	38·9	60·9	Ypsy2R GTCCGAACCCGAGCCATA	38·9	62·0
P. quercina	Yque1F GTTCGCGTCCGTGTACTTTT	50·0	60·2	Yque2R CCGTGGGTCTTCTCAGTAAAG	47·6	58·9
P. ramorum	Yram1F GACCTCTCTCTCTCTCCCTCCA	40·9	61·4	Yram2R GGGGAACGCAGACGTACAAT	45·0	62·2

[a]TM, melting temperature as calculated with PRIMER 3 software (Rozen & Skaletsky, 2000).

57·3 and 62·5°C and an AT/CG ratio between 39 and 66% (Table 4). *Phytophthora ramorum* was the only species containing intraspecific variability, but primers were designed to identify both European and American isolates.

Four or five different primers were designed for each *Phytophthora* species and tested in several combinations for the brightness of the amplified fragment and the absence of non-specific PCR bands and/or dimers. Where the selected primer set did not produce the desired level of specificity and/or sensitivity (see below) a second pair was used.

Assessment of primer specificity

Preliminary assessment of the specificity of all primers was by means of BLAST analyses, which explored available DNA sequence databases and excluded primers for which there were very similar sequences in other microrganisms. Thereafter, the primers were tested against genomic DNA

Table 4 Length of amplified fragment and levels of sensitivity achieved amplifying target DNAs with *Phytophthora*-genus-specific primers before amplification with the various species-specific primers (nested PCR) or with species-specific primers only (single round of PCR)

Target species	Specific primers	Amplified fragments (bp)	Detection limits (μL^{-1})	
			Single-round PCR	Nested-PCR
Phytophthora spp.	Yph1F-2R	≈ 470	100 pg	–
P. cactorum	Ycac1F-2R	194	10 pg	100 fg
P. cambivora	Ycam4F-3R	183	100 pg	100 fg
P. cinnamomi	Ycin3F-4R	243	100 pg	100 fg
P. citricola	Ycit1F-2R	230	10 pg	100 fg
P. europaea	Yeur1F-2R	190	10 pg	100 fg
P. ilicis	Yili1F-4R	219	10 pg	100 fg
P. inundata	Yinu2F-3R	221	10 pg	100 fg
P. kernoviae	Yptc1F-2R	247	10 pg	100 fg
P. lateralis	Ylat3F-2R	133	100 pg	100 fg
P. megasperma	Ymeg1F-2R	196	10 pg	100 fg
P. nemorosa	Ynem1F-2R	198	100 pg	100 fg
P. pseudosyringae	Ypse1F-2R	205	10 pg	100 fg
P. psychrophila	Ypsy1F-2R	169	100 pg	100 fg
P. quercina	Yque1F-2R	258	10 pg	100 fg
P. ramorum	Yram1F-2R	155	10 pg	100 fg

from 73 isolates of *Phytophthora* from across the genus (see above and Table 2) and the nine representatives of *Pythium* listed above.

Assessment of primer sensitivity

The sensitivity of each primer set was tested for the appropriate species using total DNA quantified by spectrophotometry and serially diluted with sterile water to yield final concentrations from 1 ng μL^{-1} to 100 ag μL^{-1}. Water was used as a negative control. Each set was tested alone in PCR and in nested-PCR after amplification with primer set Yph1F-Yph2R: in the latter case, 1 μL of amplified products from the first round of PCR was used as the template for the specific primers.

Amplification conditions

Reaction mixes of 15 μL comprised 1·5 μL genomic DNA (corresponding to 15 ng DNA 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 *Taq* polymerase (Taq DNA polymerase, Promega Corporation) and 6 μM primers. The sole exception was the specific primer set for *P. psychrophila*, for which the concentration of $MgCl_2$ was reduced to 0.5 mM. Common amplification conditions for all primer sets were as follows: 1 cycle of 95°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s (45 s in the case of the *Phytophthora*-genus-specific primers) 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) in TBE buffer and visualized with UV light.

Detection of target pathogens in artificially and naturally infected leaves

Five leaves each of rhododendron *(Rhododendron ponticum*), holm oak (*Quercus ilex*), beech, sweet cherry (*Prunus avium*) and birch (*Betula pendula*) were inoculated with *P. ramorum* (isolate SCRP911), *P. kernoviae* (isolate SCRP722), *P. citricola* (isolate SCRP130) or *P. quercina* (isolate SCRP541), following the procedure described by Schena *et al.* (2006). Controls comprised wounded but uninoculated leaves. DNA was extracted in triplicate from each plant and pathogen combination, and amplified with all specific primer sets.

Forty-five DNA samples extracted from naturally infected leaves according to a published protocol (Hughes *et al.*, 2006), were received from the Central Science Laboratory (CSL) of the Department for the Environment and Rural Affairs (Defra), York, UK. These samples were amplified with all specific primer sets. The leaves had been collected initially from across England by Defra's Plant Health and Seeds Inspectorate (PHSI) during an extensive survey for the presence of *P. ramorum* and *P. kernoviae*. All samples had been analysed previously at CSL by classical isolation of the two pathogens onto agar (Hughes *et al.*, 2006). The samples were provided 'blind' by CSL and the results of the previous classical analysis were only known after the present PCR analyses were completed.

Detection of target pathogens in naturally infested soil and water samples

Seven soil and four water samples were collected from four different natural ecosystems around Scotland (Table 5). Each soil sample (approximately 2 kg) was collected by digging to a depth of 15–20 cm to avoid leaf litter and consisted of 3–4 subsamples that were subsequently combined and mixed thoroughly. Water samples consisted of 10 L and were collected from rivers and streams. Two samples of water and soil, expected to be free of target phytophthoras because of their location (far from potential sources of infection) and because they always tested negative in previous trials, were collected in a field of the Scottish Crop Research Institute (SCRI) and functioned as negative controls. All samples were stored

Table 5 Results of the analysis of naturally infested soil and water samples obtained by amplifying target DNAs with *Phytophthora*-genus-specific primers before amplification with the 15 species-specific primers (nested PCR)

Location	Samples	*Phytophthora* species detected
Invergowrie (central-eastern Scotland)	Soil 1	*P. cambivora, P. citricola*
	Soil 2	*P. cambivora*
	Soil 3	None
	Soil 4	*P. cinnamomi, P. citricola*
Dunkeld (central Scotland)	Soil 5	*P. citricola*
	Water 1	None
	Water 2	*P. citricola, P. inundata*
Loch Lomond (central-western Scotland)	Soil 6	None
	Soil 7	*P. inundata*
	Water 3	*P. citricola, P. inundata, P. europaea, P. pseudosyringae*
Clyde valley (southwest Scotland)	Water 4	None

at 5°C while waiting to be analysed. When a positive result was obtained, the identity of the amplified fragments was confirmed by sequencing. Selected fragments were purified and directly sequenced with the same primers used for amplification (Schena & Cooke, 2006).

Results

DNA extractions and primer specificity and sensitivity

The DNA extraction protocol yielded 10–20 μg of high quality genomic DNA (260/280 ratio ranging from 1·8 to 2·0) from pure cultures. The protocols for plant tissue and soil and water samples enabled the extraction of total nucleic acid suitable for PCR amplification within 3–4 h, with average yields of 1–3 μg g^{-1} plant material, 0·5–1·5 μg g^{-1} soil, and 0·05–0·5 μg L^{-1} water.

The *Phytophthora*-genus-specific primers Yph1F-Yph2R amplified a single PCR band with all *Phytophthora* species tested, but none with any *Pythium* species (Table 6). Amplified fragments ranged from 419 to 478 bp, depending on *Phytophthora* species, and comprised two small portions of exons 3 and 6 and the complete exons and introns between these flanking regions (Fig. 1). With two exceptions, the species-specific primers were highly specific, only amplifying single PCR bands, ranging from 133 to 258 bp, from their target *Phytophthora* (Table 4). The two exceptions were *P. cactorum*/*P. idaei* and *P. ilicis*/*P. nemorosa*. Within each of these pairs, the species could not be separated because relevant sequences in the *Ypt*1 gene were identical.

The detection limit of a single round of PCR amplifications ranged from 100 to 10 pg of DNA, depending on *Phytophthora* species (Table 4). In all cases the detection limit was increased to 100 fg in nested PCR after a first amplification with primer set Yph1F-Yph2R. The same detection limits were obtained in repeated experiments using different DNA extractions.

Tests with artificially and naturally infected leaves

Phytophthora ramorum, *P. kernoviae*, *P. citricola* and *P. quercina* produced lesions on artificially inoculated leaves of oak, cherry and beech, but only *P. kernoviae* and *P. ramorum* were able to infect rhododendron leaves, and *P. citricola* and *P. ramorum* birch leaves.

The universal primers and probe (Schena *et al*., 2006) amplified DNA from all leaf samples, including uninoculated controls: Ct values ranged from 11·1 to 18·2 (data not shown). Specific primers for *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* generated bands of the expected size from leaves inoculated with the corresponding pathogen (Fig. 2), but no bands were obtained from leaves inoculated with non-target *Phytophthora* species, or from healthy controls. Similarly, the specific primers for the 11 other *Phytophthora* species not included in the inoculation experiment did not generate bands from any of the inoculated or control leaves.

Of the 45 DNA samples from naturally infected leaves analysed with all species-specific primers, 19 were positive for *P. ramorum* and eight for *P. kernoviae*. No amplification was obtained with any other species-specific primers (data not shown). The positive samples were those that had also proved positive in classical isolation onto selective media by CSL. Once again, the universal probe and primers (Schena *et al*., 2006) confirmed that all extracted DNAs were of good quality and amplifiable (Ct values ranged from 13·8 to 19·1).

Detection of target pathogens in naturally infested soils and water

Results with naturally infested soil and water samples are given in Table 5. No positives were detected by single amplification with species-specific primers. However, after nested-PCR, eight out of 14 were positive for at least one *Phytophthora* species. The most common species was *P. citricola* (five positive samples), but others detected included *P. inundata* (three positives) *P. cambivora* (two) and *P. europaea* and *P. pseudosyringae* (one each) (Table 5). No amplification was obtained with any primer pair in the negative water and soil controls.

In all cases, sequencing of the amplified fragments yielded a sequence identical to that of the target species (data not shown). All extracted DNAs were of good

Table 6 Results of specificity tests conducted with *Phytophthora*-genus-specific and species-specific primers

	Specific primers															
Target species	Yph1F-2R	Ycac1F-2R	Ycam4F-3R	Ycin3F-4R	Ycit1F-2R	Yeur1F-2R	Yili1F-4R	Yinu2F-3R	Yptc1F-2R	Ylat3F-2R	Ymeg1F-2R	Ynem1F-2R	Ypse1F-2R	Ypsy1F-2R	Yque1F-2R	Yram1F-2R
P. alni subsp. *alni**	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. alni subsp. *multiformis**	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. alni subsp. *uniformis**	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. boehmeriae	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*P. cactorum**	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*P. cambivora**	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
P. capsici	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*P. cinnamomi**	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
*P. citricola**	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
P. citrophthora	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. cryptogea	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. drechsleri	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. erythroseptica	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. europaea	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
P. fragariae var. *fragariae**	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. fragariae var. *rubi**	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*P. idaei**	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*P. ilicis**	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
P. infestans	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. insolita	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*P. inundata**	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
P. katsurae	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*P. kernoviae**	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
P. lateralis	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
P. medicaginis	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. megasperma	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
P. nemorosa	+	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–
P. nicotianae	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. palmivora	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. pistaciae	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*P. pseudosyringae**	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
P. psychrophila	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
*P. quercina**	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
*P. ramorum**	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
P. sojae	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium pyrilobum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium catenulatum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium torulosum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium intermedium	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium dissotocum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium aphanidermatum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium ultimum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium undulatum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium splendens	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

*When more then one isolate per species of *Phytophthora* was analysed (see Table 2) results were identical.

quality and amplifiable (Ct values ranging from 19·5 to 25·0), as assessed by the universal probe and primers.

Discussion

The aim of this research was to develop a PCR-based 'molecular tool box' that could identify 15 *Phytophthora* species important in declines of forests and other natural ecosystems. Although, several molecular detection methods have been used to detect forest phytophthoras (Table 1), this is the first to report the development of a comprehensive set for the detection of such a large number of species. All the primers were developed around a fragment of the *Ypt*1 gene, which is characterized by the

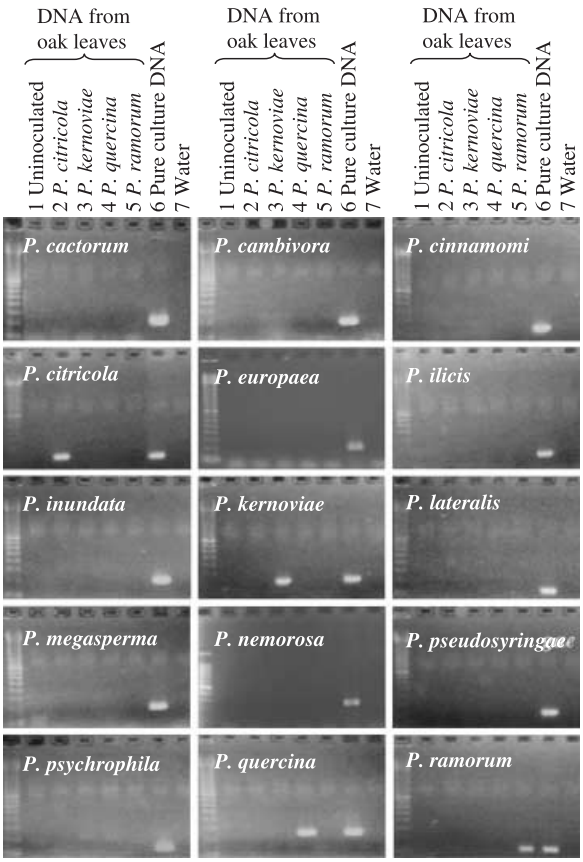

Figure 2 PCR amplification products of all specific primer pairs included in the 'molecular tool box' (Table 3) when tested against DNA extracted from oak leaves inoculated with *Phytophthora citricola*, *P. kernoviae*, *P. quercina* or *P. ramorum* (lanes 2, 3, 4 and 5, respectively) or uninoculated (lane 1). Additional positive and negative controls comprised DNA extracted from a pure culture of each target species (lane 6) and water (lane 7). In each gel, the first lane on the left contains the 100-bp DNA ladder.

presence of conserved coding regions flanking very variable introns (Schena *et al*., 2006). The highly polymorphic nature of these introns was essential to differentiate closely related species which have almost identical ITS regions (Cooke *et al*., 2000; Kroon *et al*., 2004). The same target region was used as a molecular marker to identify *P. fragariae* (Ioos *et al*., 2006) and to develop a multiplex real-time PCR detection method for *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* (Schena *et al*., 2006). The analysis of the sequences of the *Ypt*1 gene from different isolates of the same species (Table 2) showed the absence of intraspecific polymorphism that could cause problems for diagnostic assays. For some species only single isolates were analysed and thus the presence of polymorphic nucleotides in other isolates from different geographic origin cannot be completely ruled out. However, the sequence data available to date are sufficient to demonstrate a substantial intraspecific conservation of the *Ypt*1 gene (Schena *et al*., 2006). A few polymorphic nucleotides were identified between the European and American isolates of *P. ramorum*, but this is not surprising since combined microsatellite, sequencing and morphological analyses suggested distinct evolutionary lineages for European and American populations (Ivors *et al*., 2006). Nevertheless, primers designed in the present study and those reported in Schena *et al*. (2006) were designed to detect all *P. ramorum* isolates.

The primers designed in this study proved highly specific, as witnessed by the absence of cross-reactions with DNA from a large number of isolates: 73 isolates of *Phytophthora* (35 species) and nine species of *Pythium*. Specificity was confirmed by BLAST analysis and by the absence of amplification from non-infected samples of leaves and from non-infested soil and water samples. Exceptions were primers designed for *P. cactorum* and *P. ilicis*, which cross-reacted with *P. idaei* and *P. nemorosa*, respectively. This result was predictable, since the species in each of the relevant pairs are very closely related, as demonstrated by the analysis of a number of mitochondrial and genomic genes, including the ITS regions (Cooke *et al*., 2000; Martin & Tooley, 2003; Kroon *et al*., 2004). *Phytophthora cactorum* and *P. idaei* differed by only three nucleotides in the region of the *Ypt*1 gene, which was the basis of the *Ypt*1 primers. More differences were found between the *Ypt*1 gene sequence of *P. ilicis* and *P. nemorosa* and these were exploited in the design of specific primers for *P. nemorosa*. However, the use of the same target region to design specific primers for *P. ilicis* was not possible because of its sequence identity with two other closely related species (*P. pseudosyringae* and *P. psychrophila*). Other regions of the *Ypt*1 gene could provide additional potential for species discrimination. Nevertheless, having primer sets that can discriminate *P. cactorum* and *P. idaei*, and *P. ilicis* and *P. nemorosa*, from other *Phytophthora* spp. is itself an advance on what has been achieved with less variable target genes.

In the present study, the only important forest *Phytophthora* sp. for which specific primers were not developed was *P. alni*. The genomic instability and complex genetics of the hybrid *P. alni* (Brasier *et al*., 2004b) represented a particular challenge. Unsurprisingly, a relatively high level of polymorphism in the *Ypt*1 gene sequence of *P. alni* subsp. *alni* and *P. alni* subsp. *multiformis* was observed (data not shown). Specific SCAR primers were developed for *P. alni* after RAPD-PCR analyses of a number of isolates (De Merlier *et al*., 2005). A similar development of the *Ypt*1 gene to identify all *P. alni* subspecies collectively and/or specific subspecies may be possible, but only after analysis of large numbers of sequences from many different isolates. Such analysis would also shed further light on the ongoing evolution of this species complex (Brasier *et al*., 2004b).

A single amplification with species-specific primers was specific and sensitive enough to detect the target species in leaves naturally and/or artificially infected with *P. ramorum, P. kernoviae, P. citricola* and *P. quercina*. Although leaves infected with the other 11 target phytophthoras were not tested in this study, it is considered that a single

PCR amplification with the appropriate primers would be sufficient also to detect each of them, considering the similar sensitivities of all the primer sets when tested on DNA from pure cultures. The only species detected in naturally infected leaves were *P. ramorum* and *P. kernoviae*, not surprising considering that the leaves had been sampled on the basis of symptoms indicating infection by these two species. Nevertheless, the PCR results were in complete agreement with those obtained previously at CSL by classical isolation on selective media and indicated clearly the potential of PCR detection in confirming species identity in such disease surveys.

In line with previous reports (Vettraino *et al*., 2002, 2005), the 'molecular tool box' developed in this study enabled the detection of *Phytophthora* species in soil and water in environments in which plant infection was not apparent. In Scotland, the commonest species detected were *P. citricola* and *P. inundata*. Unlike leaf samples, soil and water samples required a nested approach to achieve appropriate levels of sensitivity, not unexpectedly given that the concentration of *Phytophthora* in soil and water is probably nearly always under the detection limit of a single PCR amplification (Ippolito *et al*., 2004). Unlike rDNA genes, which exist in multiple copies, the *Ypt*1 gene is only single copy (Chen & Roxby, 1996). However, one round of amplification with *Ypt*1 primers for each individual species detected 10–100 pg target DNA μL^{-1}, a sensitivity that was increased to 100 fg μL^{-1} in nested PCR with the genus-specific primers. The lower level of 100 fg μL^{-1} corresponds to one or a few copies of the target gene (Schena *et al*., 2006), making the detection of a single propagule by PCR theoretically possible. Although no specific tests were conducted in the present study, the high sensitivity of the nested approach indicates that this method is likely to be appropriate to detect early infections before the appearance of symptoms.

One great advantage of the *Ypt*1 gene is that its structure (alternate conserved and variable regions) has enabled the development of nested PCR with a first round using the genus-specific primers Yph1F-Yph2R (Schena *et al*., 2006), and a second with species-specific primers. This allows the use of a common amplified product from the first amplification as template for all the specific primers, with significant reductions of time and cost. The utility of such an approach was enhanced by designing primers with the same amplification conditions for the 'molecular tool box'. However, the use of nested PCR implies a greater risk of false positives arising from cross contaminations, as well as increasing the time and labour requirements of the procedure.

In conclusion a 'molecular tool box' developed for the identification of a number *Phytophthora* species was specific and sensitive enough to detect target species in infected leaves and infested soil and water samples. The 'tool box' represents an important method for detailed surveys of *Phytophthora* species in a variety of habitats and for tracking their movement locally in soil and water and further afield in plants. Other *Phytophthora* species can be included in future using primers designed on the same target gene, and furthermore the small sizes of the PCR fragments (133–258 bp) are particularly appropriate for real-time PCR (Schena *et al*., 2004).

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References

Böhm J, Hahn A, Schubert R, *et al*., 1999. Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus *Glomus mosseae* and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. *Journal of Phytopathology* **147**, 409–16.

Brasier C, Denman S, Brown A, Webber J, 2004a. Sudden oak death (*Phytophthora ramorum*) discovered on trees in Europe. *Mycological Research* **108**, 1107–10.

Brasier CM, Cooke DEL, Duncan JM, 1999. Origin of a new *Phytophthora* pathogen through interspecific hybridization. *Proceedings of the National Academy of Sciences, USA* **96**, 5878–83.

Brasier CM, Sánchez-Hernandez E, Kirk SA, 2003. *Phytophthora inundata* sp. nov., a part heterothallic pathogen of trees and shrubs in wet or flooded soils. *Mycological Research* **107**, 477–84.

Brasier CM, Kirk SA, Delcan J, Cooke DEL, Jung T, Man In't Veld WA, 2004b. *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. *Mycological Research* **108**, 1172–84.

Brasier CM, Beales PA, Kirk SA, 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 Britain. *Mycological Research* **109**, 853–9.

Brown AV, Brasier CM, 2007. Colonization of tree xylem by *Phytophthora ramorum*, *P. kernoviae* and other *Phytophthora* species. *Plant Pathology* **56**, 227–41.

Causin R, Scopel C, Grendene A, Montecchio L, 2005. An improved method for the detection of *Phytophthora cactorum* (L.C.) Schröeter in infected plant tissues using SCAR markers. *Journal of Plant Pathology* **87**, 25–35.

Chen Y, Roxby R, 1996. Characterization of a *Phytophthora infestans* gene involved in the vesicle transport. *Gene* **181**, 89–94.

Coakley SM, Scherm H, Chakraborty S, 1999. Climate change and plant disease management. *Annual Review of Phytopathology* **37**, 399–426.

Coelho AC, Cravador A, Bollen A *et al*., 1997. Highly specific and sensitive non-radioactive molecular identification of *Phytophthora cinnamomi*. *Mycological Research* **101**, 1499–507.

Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM, 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genetics and Biology* **30**, 17–32.

Cooke DEL, Jung T, Williams NA, Schubert R, Oßwald W, Duncan JM, 2005. Genetic diversity of European populations of the oak fine-root pathogen *Phytophthora quercina*. *Forest Pathology* **35**, 57–70.

Cooke DEL, Schena L, Cacciola SO, 2007. Tools to detect, identify and monitor *Phytophthora* species in natural ecosystems. *Journal of Plant Pathology* **89**, 13–28.

Cullen DW, Lees AK, Toth IK, Duncan JM, 2001. Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and potato tubers. *European Journal of Plant Pathology* **107**, 387–98.

De Merlier D, Chandelier A, Debruxelles N, *et al*., 2005. Characterization of alder *Phytophthora* isolates from Wallonia and development of SCAR primers for their specific detection. *Journal of Phytopathology* **153**, 99–107.

Hansen EM, Reeser PW, Davidson JM, *et al*., 2003. *Phytophthora nemorosa*, a new species causing cankers and leaf blight of forest trees in California and Oregon, USA. *Mycotaxon* **88**, 129–38.

Hayden KJ, Rizzo D, Tse J, Garbelotto M, 2004. Detection and quantification of *Phytophthora ramorum* from California forests using a real-time polymerase chain reaction assay. *Phytopathology* **94**, 1075–83.

Hughes KJD, Griffin RL, Tomlinson JA, Boonham N, Inman AJ, Lane CR, 2006. Development of a one-step real-time PCR assay for diagnosis of *Phytophthora ramorum*. *Phytopathology* **96**, 975–81.

Ioos R, Husson C, Andrieux A, Frey P, 2005. SCAR-based PCR primers to detect the hybrid pathogen *Phytophthora alni* and its subspecies causing alder disease in Europe. *European Journal of Plant Pathology* **112**, 323–35.

Ioos R, Laugustin L, Schenck N, Rose S, Husson C, Frey P, 2006. Usefulness of single copy genes containing introns in *Phytophthora* for the development of detection tools for the regulated species *P. ramorum* and *P. fragariae*. *European Journal of Plant Pathology* **116**, 171–6.

Ippolito A, Schena L, Soleti Logorio V, Yaseen T, Nigro F, 2004. Real-time detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soils. *European Journal of Plant Pathology* **110**, 833–43.

Ivors K, Garbelotto M, Vries IDE, *et al*., 2006. Microsatellite markers identify three lineages of *Phytophthora ramorum* in US nurseries, yet single lineages in US forest and European nursery populations. *Molecular Ecology* **15**, 1493–505.

Jung T, Blaschke H, Oßwald W, 2000. Involvement of soilborne *Phytophthora* species in Central European oak decline and the effect of site factors on the disease. *Plant Pathology* **49**, 706–18.

Jung T, Hansen EM, Winton L, Oßwald W, Delatour C, 2002. Three new species of *Phytophthora* from European oak forests. *Mycological Research* **106**, 397–411.

Jung T, Nechwatal J, Cooke DEL, *et al*., 2003. *Phytophthora pseudosyringae* sp. nov., a new species causing root and collar rot of deciduous tree species in Europe. *Mycological Research* **107**, 772–89.

Jung T, Hudler GW, Jensen-Tracy SL, Griffiths HM, Fleischmann F, Osswald W, 2005. Involvement of *Phytophthora* species in the decline of European beech in Europe and the USA. *Mycologist* **19**, 159–66.

Jurskis V, 2005. Eucalypt decline in Australia, and a general concept of tree decline and dieback. *Forest Ecology and Management* **215**, 1–20.

Kong P, Hong CX, Richardson PA, 2003. Rapid detection of *Phytophthora cinnamomi* using PCR with primers derived from the *Lpv* putative storage protein genes. *Plant Pathology* **52**, 681–93.

Kroon LPNM, Bakker FT, van den Bosch GBM, Bonants PJM, Flier WG, 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology* **41**, 766–82.

Luque J, Parladé J, Pera J, 2002. Seasonal changes in susceptibility of *Quercus suber* to *Botryosphaeria stevensii* and *Phytophthora cinnamomi*. *Plant Pathology* **51**, 338–45.

Martin FN, Tooley PW, 2003. Phylogenetic relationships of *Phytophthora ramorum*, *P. nemorosa*, and *P. pseudosyringae*, three species recovered from areas in California with sudden oak death *Mycological Research* **107**, 1379–91.

Martin FN, Tooley PW, 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**, 621–31.

Nechwatal J, Schlenzing A, Jung T, Cooke DEL, Duncan JM, Oßwald WF, 2001. A combination of baiting and PCR techniques for the detection of *Phytophthora quercina* and *P. citricola* in soil samples from oak stands. *Forest Pathology* **31**, 85–97.

Rizzo DM, Garbelotto M, Hansen EM, 2005. *Phytophthora ramorum*: integrative research and management of an emerging pathogen in California and Oregon forests. *Annual Review of Phytopathology* **43**, 309–35.

Rozen S, Skaletsky HJ, 2000. Primer 3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Totowa, NJ, USA: Humana Press, 365–86.

Schena L, Cooke DEL, 2006. Assessing the potential of regions of the nuclear and mitochondrial genome to develop a 'molecular tool box' for the detection and characterization of *Phytophthora* species. *Journal of Microbiological Methods* **67**, 70–85.

Schena L, Nigro F, Ippolito A, Gallitelli D, 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology* **110**, 893–908.

Schena L, Hughes KJD, Cooke DEL, 2006. Detection and quantification of *Phytophthora ramorum*, *P. kernoviae*, *P. citricola*, and *P. quercina* in symptomatic leaves by multiplex real-time PCR. *Molecular Plant Pathology* **7**, 365–79.

Schubert R, Bahnweg G, Nechwatal J, *et al*., 1999. Detection and quantification of *Phytophthora* species which are associated with root-rot diseases in European deciduous forests by species-specific polymerase chain reaction. *Forest Pathology* **29**, 169–88.

Tooley PW, Kyde KL, Englander L, 2004. Susceptibility of selected ericaceous ornamental host species to *Phytophthora ramorum*. *Plant Disease* **88**, 993–9.

Tooley PW, Martin FN, Carras MM, Frederick RD, 2006. Real-time fluorescent polymerase chain reaction detection of *Phytophthora ramorum* and *Phytophthora pseudosyringae* using mitochondrial gene regions. *Phytopathology* **96**, 336–45.

Vettraino AM, Barzanti GP, Bianco MC, *et al*., 2002. Occurrence of *Phytophthora* species in oak stands in Italy and their association with declining oak trees. *Forest Pathology* **32**, 19–28.

Vettraino AM, Morel O, Perlerou C, Robin C, Diamandis S, Vannini A, 2005. Occurrence and distribution of *Phytophthora* species in European chestnut stands, and their association with ink disease and crown decline. *European Journal of Plant Pathology* **111**, 169–80.

Winton LM, Hansen EM, 2001. Molecular diagnosis of *Phytophthora lateralis* in trees, water, and foliage baits using multiplex polymerase chain reaction. *Forest Pathology* **31**, 275–83.